

Table 1 Summary of peripheral-type and central-type benzodiazepine receptor characteristics

	Peripheral-type	Central-type
Tissue distribution	Ubiquitously distributed in peripheral tissues (especially steroidogenic) as well as in glia of CNS	Neuronal
Subcellular localisation	Mitochondrial outer membrane	Plasma membranes
Synthetic ligands	Isoquinolines (e.g. PK 11195), benzodiazepines (e.g. Ro5-4864, species-dependent, GABA-insensitive)	Benzodiazepines (e.g. clonazepam, GABA-sensitive)
Endogenous ligands	diazepam binding inhibitor (DBI)	DBI
Molecular components	Isoquinoline binding protein (18kDa), mitochondrial VDAC (32kDa), ANC (30kDa)	Heterogenous $\alpha$ , $\gamma$ subunits of GABA <sub>A</sub> receptor
Effector mechanism	cholesterol transport?	Regulates chloride flux by modulating GABA binding to GABA <sub>A</sub> receptor

Compound	% Apoptosis
Control	1.1 ± 1.2
PBOX 3	23.5 ± 2.1
PBOX 4	42.1 ± 0.6
PBOX 5	27.6 ± 4.2
PBOX 6	38.6 ± 4.6
PBOX 7	40.7 ± 2.5
PBOX 8	38.6 ± 3.1
PBOX 9	40.4 ± 3.0
PBOX 12	27.1 ± 2.7
PBOX 24	7.0 ± 1.2
PBOX 25	19.2 ± 3.0
PBOX 26	7.8 ± 2.3
PBOX 27	11.8 ± 3.2
PBOX 28	31.1 ± 1.7
PBOX 30	33.1 ± 1.6

#### Effect of PBOX compounds on apoptosis in HL-60 cells

HL-60 cells were seeded at a density of  $3 \times 10^5$  cell/ml into 24-well plates and treated for 16 h with the various PBOX compounds (10 $\mu$ M) listed above. Control wells contained 1% (v/v) ethanol. Percent apoptosis was determined after cytopinning and staining the cells. The values represent the mean  $\pm$  range of two experiments.

TABLE 2

TABLE 3

High affinity binding of pyrrolobenzoxazepines to homogenates of HL-60 cells

Compound	Inhibition of [ $^3\text{H}$ ]PK 11195 binding to the PBR, $K_i$ value (nM)
PBOX-1	$3.4 \pm 0.2$
PBOX-2	$1.2 \pm 1.1$
PBOX-3	$6.0 \pm 1.9$
PBOX-4	$1.9 \pm 2.3$
PBOX-5	$1.1 \pm 0.9$
PBOX-6	$6.7 \pm 1.7$
PBOX-7	$1.7 \pm 0.7$

Homogenates (50  $\mu\text{g}$  of protein) of HL-60 cells were assayed for specific binding of [ $^3\text{H}$ ]PK 11195 binding (2nM) in the presence and absence of the indicated unlabelled displacing compound (0-1  $\mu\text{M}$ ). The amount of radioactivity bound in the presence of the displacer was expressed as a percentage of control binding and the values represent the mean  $\pm$  SEM of triplicate determinations from one representative experiment performed at least twice. The  $K_i$  values were obtained as described in Methods section.

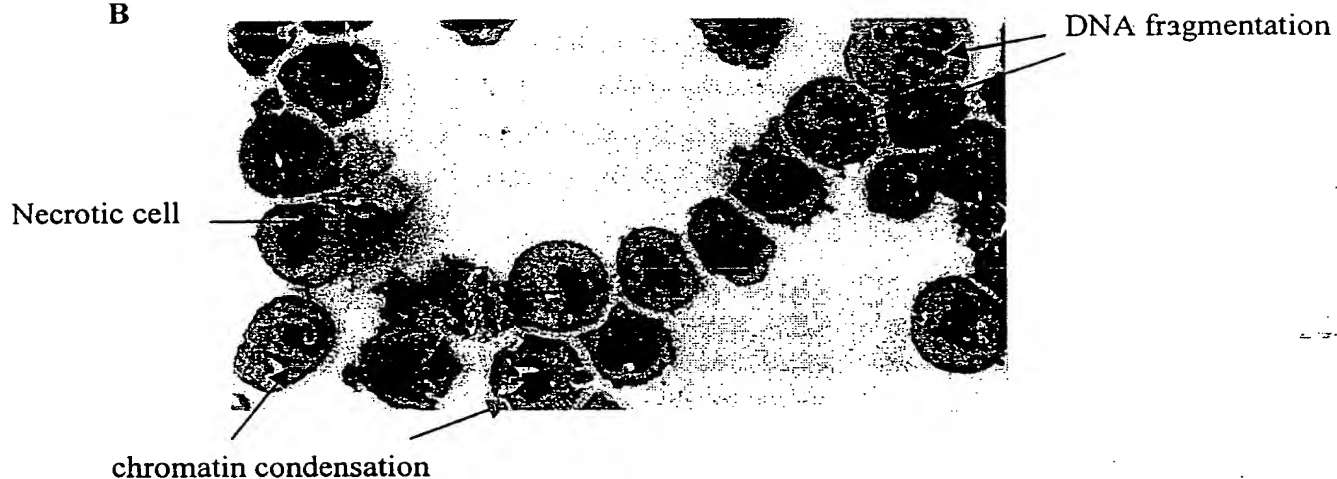
Normal Bone Marrow	0.5% Ethanol	10 $\mu$ M PBOX-6
1	10	10
2	13	16
3	17	16
4	22	22
5	22	19
6	15	11
7	16	20
8	14	17
9	21	18
10	17	20
Mean	17	16.9
SD	4.45	3.87

**Table 4 PBOX-6 has no significant effect on normal bone marrow progenitor cells.** Mononuclear cells were isolated as described in materials and methods and treated with either ethanol (0.5% v/v) or PBOX-6 (10 $\mu$ M) for 72 hours. Cells were plated in triplicate onto semi-solid medium at  $1 \times 10^5$  cells per sample and incubated at 37°C. After 14 days incubation, colonies were scored. A colony was scored as a cluster containing >40 cells, which originate from one stem cell.

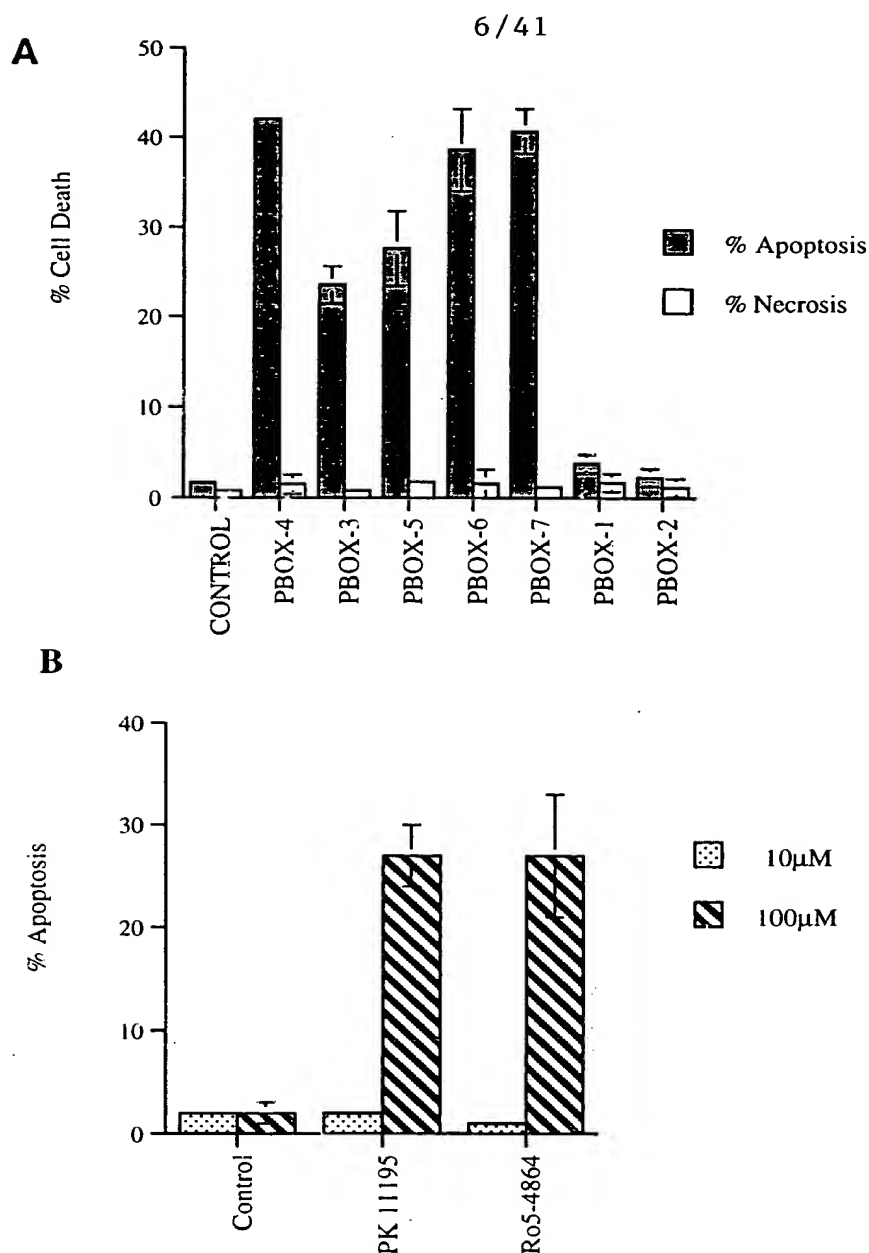
A



B

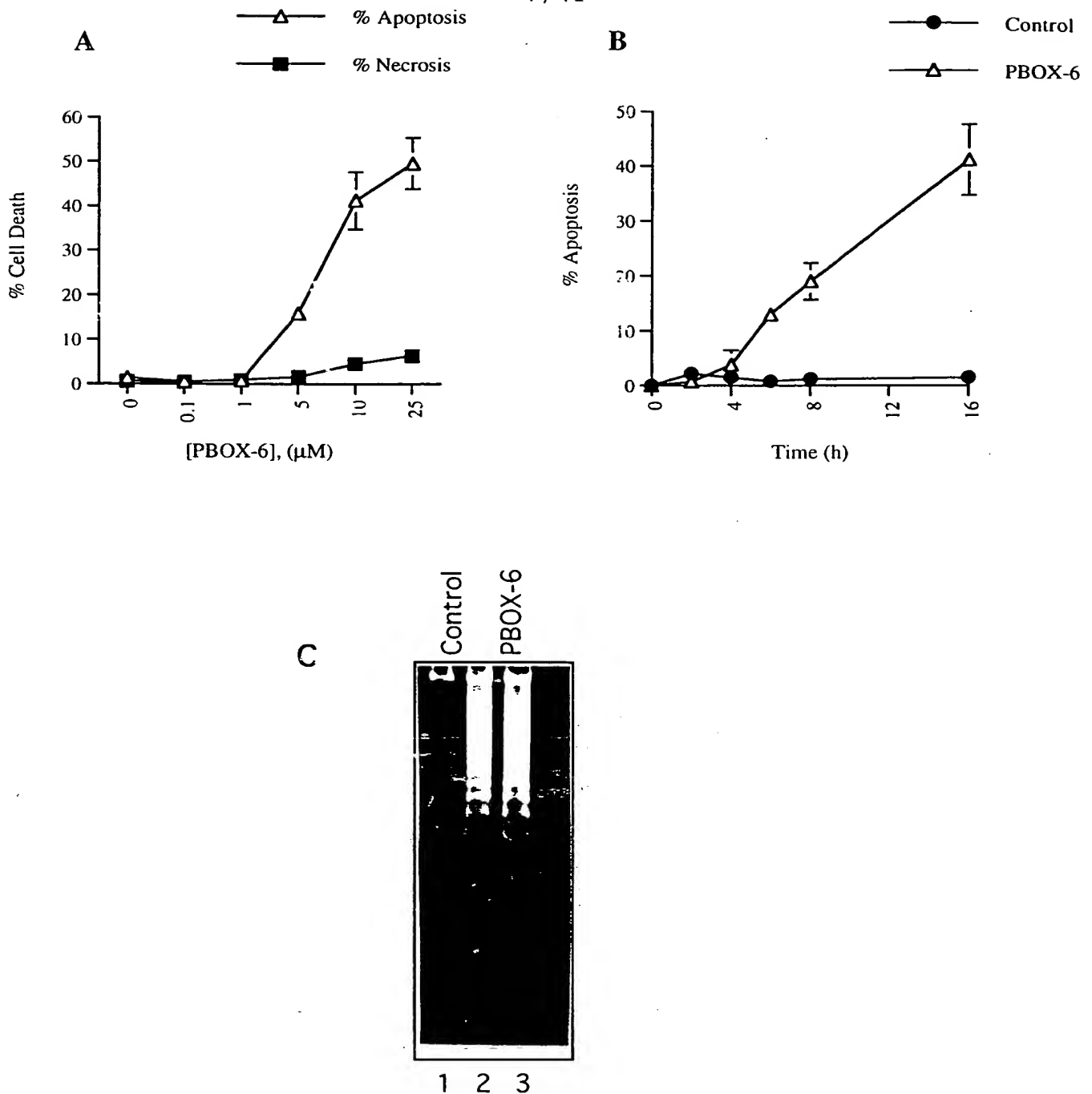


**Fig.1. Morphological features of HL-60 cells undergoing apoptosis following treatment with PBOX6.** Microscopic analysis of HL-60 cells was performed on cytospin samples. Vehicle (1% ethanol) treated cells (A) are characterised by a continuous plasma membrane and an intact nucleus. PBOX-6 treated cells (B) display the morphological features of apoptosis which includes chromatin condensation and DNA fragmentation.



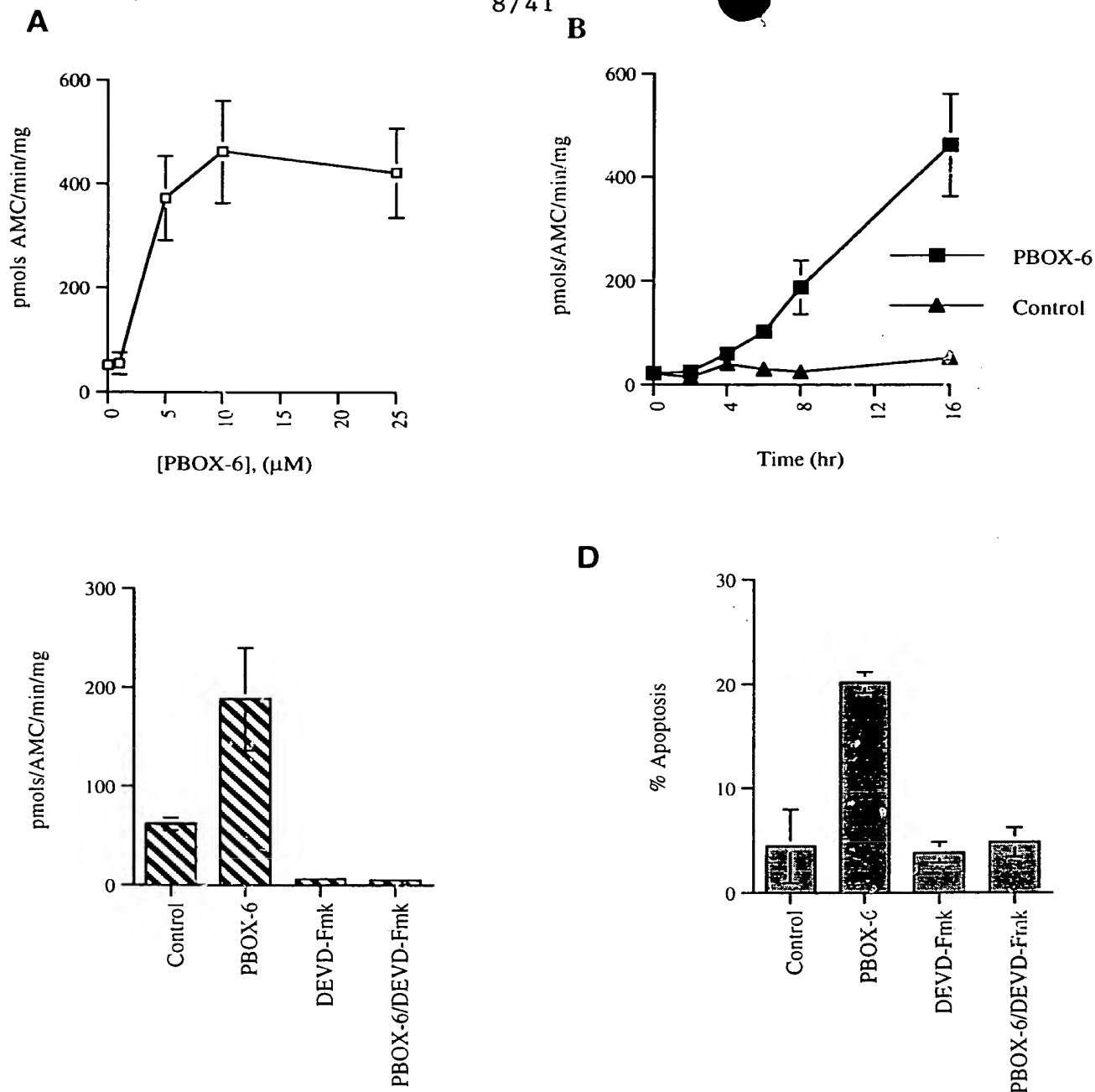
**Fig. 2. Some pyrrolo-1,5-benzoxazepines and other PBR ligands induce apoptosis in HL 60 cells.**

HL 60 cells were seeded at a density of  $3 \times 10^5$  cells/ml and were incubated with either (A), one of the indicated PBOX drugs, each at a final concentration of 10µM or (B), PK 11195 or Ro5-4864 at a concentration of either 10µM or 100µM. The control wells in each case contained 1% (v/v). After 16h the percent apoptosis and necrosis was determined by cytospinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the means  $\pm$  SEM for three separate experiments.



**Fig. 3. PBOX-6-induced apoptosis in HL 60 cells is dose- and time-dependent and results in DNA fragmentation.**

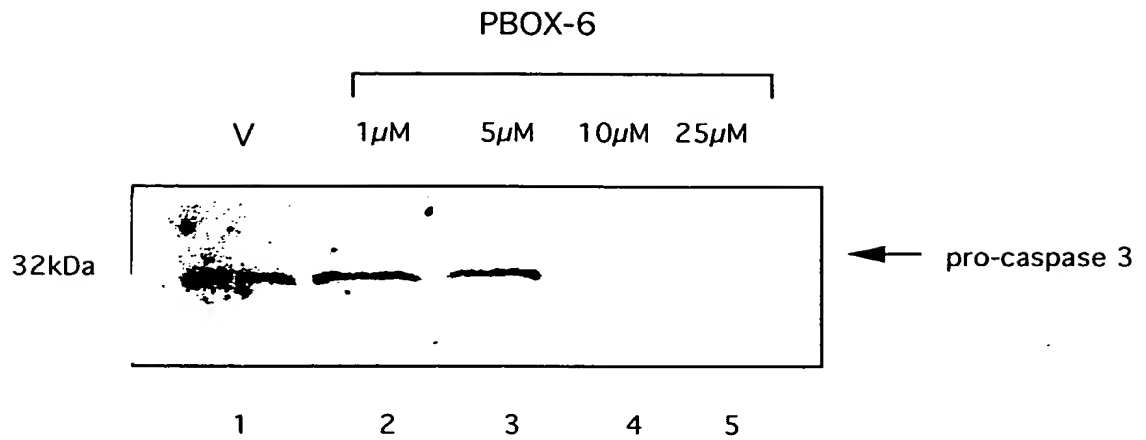
HL 60 cells were seeded at a density of  $3 \times 10^5$  cells/ml and were treated with either (A), a range (0-50  $\mu$ M) of concentrations of PBOX-6 for 16 hours or (B), one concentration of PBOX-6 (10  $\mu$ M) for a period of 2,4,6,8 and 16 hours. The percent apoptosis and necrosis was determined by cytospinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the means  $\pm$  SEM for three separate experiments. (C) DNA isolated from HL-60 cells, treated for 24 hours either with control (0.5% (v/v) ethanol) or PBOX-6 (10  $\mu$ M) in duplicate, was analysed by gel electrophoresis.



**Fig. 4. PBOX-6 induces apoptosis through activation of caspase 3-like proteases.**

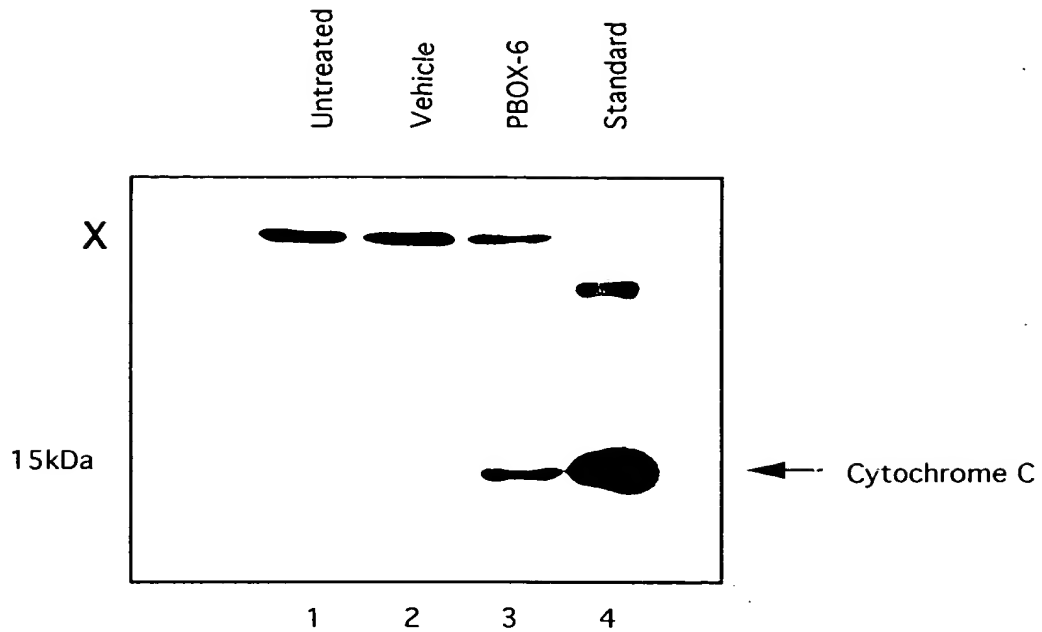
HL-60 cells were seeded at a density of  $3 \times 10^5$  cells/ml and were treated with either (A) a range (0-50 $\mu$ M) of concentrations of PBOX-6 for 16 hours or (B) one concentration of PBOX-6 (10 $\mu$ M) for a period of 2,4,6,8 and 16 hours or (C and D) pre-treated with z-DEVD-fmk (200 $\mu$ M) for 1 h followed by treatment with PBOX-6 for a further 8h. Cytosolic extracts were prepared and assayed for caspase 3-like protease activity as described in the Methods section. The percent apoptosis and necrosis was determined by cytopinning and staining the cells using the RapiDiff kit. Values represent the means  $\pm$  SEM of three separate experiments.





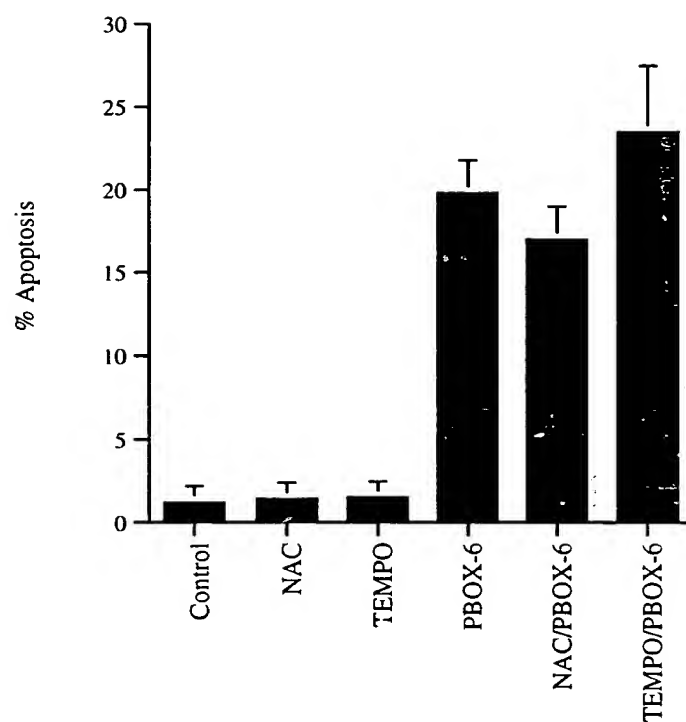
**Fig. 5. Disappearance of pro-caspase 3 in HL-60 cells in response to PBOX-6 treatment.**

Extracts from HL-60 cells were prepared, treated with either vehicle (ethanol) or a range of concentrations (1 $\mu$ M, 5 $\mu$ M, 10 $\mu$ M and 25 $\mu$ M) of PBOX-6 for 16 h. Samples (30 $\mu$ g of protein) were resolved by SDS-PAGE and probed for pro-caspase 3. Results are representative of at least two separate experiments. V, vehicle.



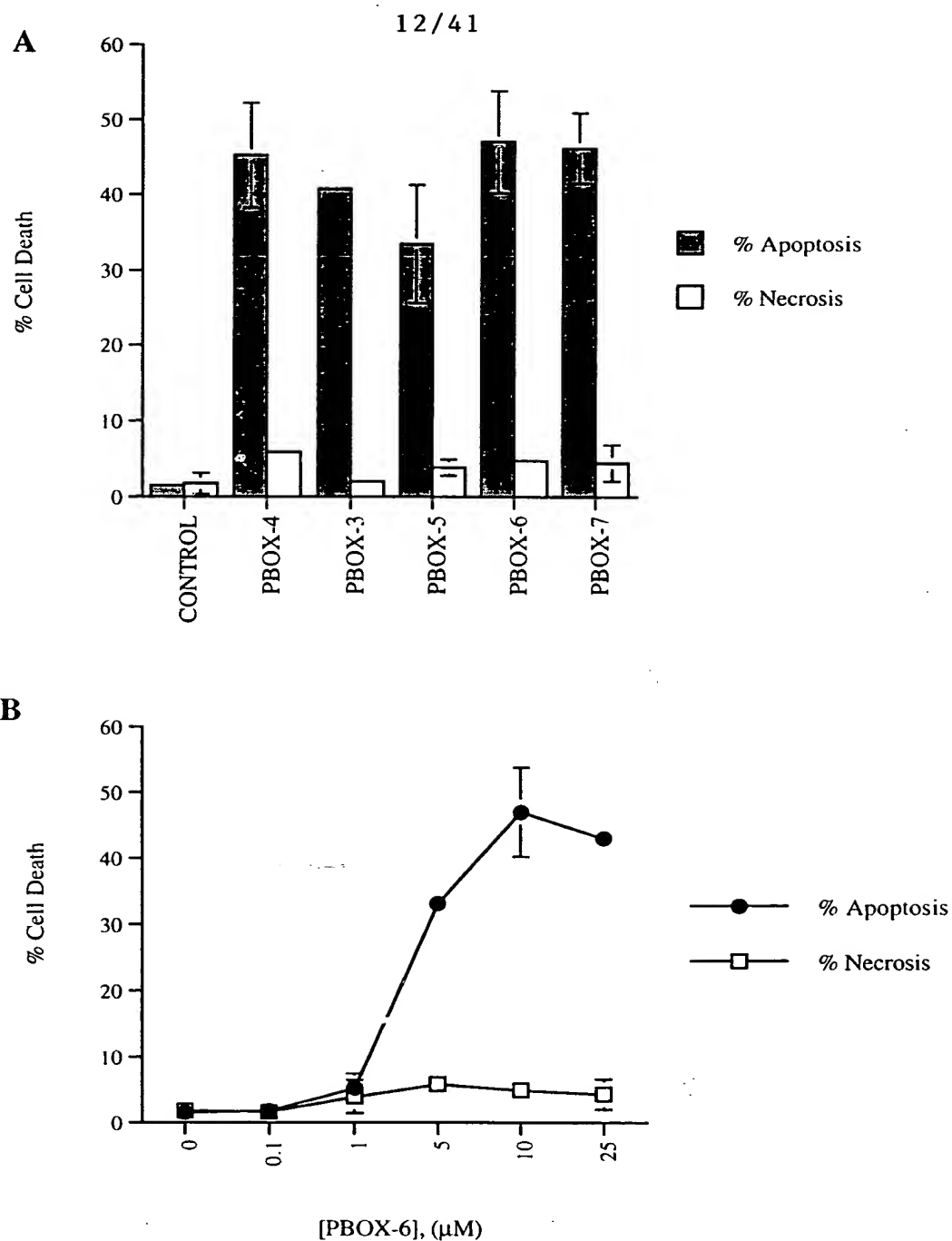
**Fig. 6. Accumulation of Cytochrome C in response to PBOX-6.**

Cytosols from HL-60 cells, were prepared as described in Methods section treated with either control (untreated), vehicle (ethanol) or PBOX-6 (10 $\mu$ M) for 16 h. Samples (30 $\mu$ g of protein) were resolved by SDS-PAGE and probed for Cytochrome C as described in the Methods section. The arrow denotes the position of Cytochrome C, horse Cytochrome C being used as a standard. The upper band in lane 4 is due to a dimer of the standard Cytochrome C sample. The X denotes a protein band that cross-reacted with the antibody as previously described (Liu et al., 1996). Results are representative of at least two separate experiments.



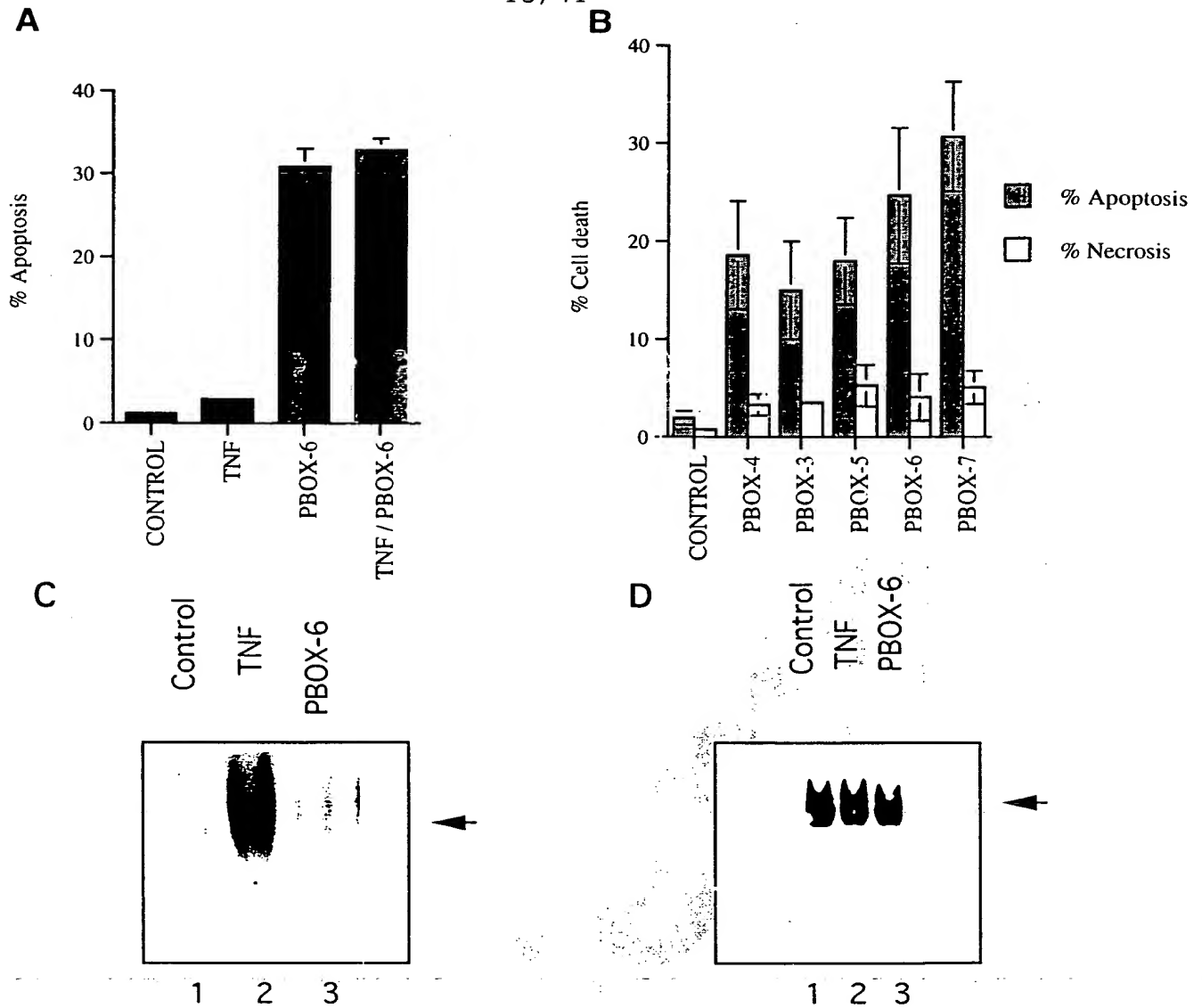
**Fig. 7. N-acetylcysteine or TEMPO pre-treatment does not protect HL-60 cells against PBOX-6-induced apoptosis.**

HL-60 cells were seeded at a density of  $3 \times 10^5$  cells/ml, and were pre-treated with either N-acetylcysteine (NAC) (5mM) or TEMPO (1 $\mu$ M) for 30 min followed by treatment with PBOX-6 for a further 8h. Percent apoptosis was determined by cytopinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the means  $\pm$  range of two separate experiments.



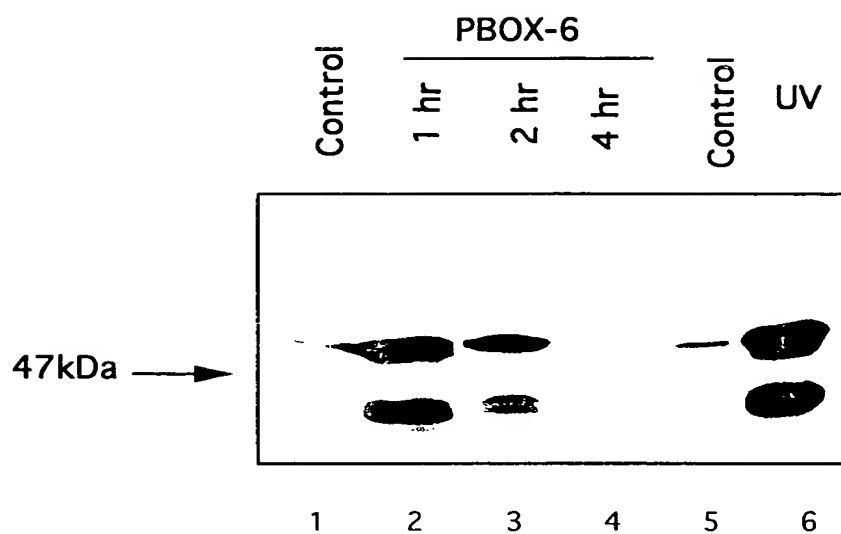
**Fig. 8. Pyrrolo-1,5-benzoxazepines induce apoptosis in Jurkat cells.**

Jurkat cells were seeded at a density of  $3 \times 10^5$  cells/ml and were incubated with (A) either one of the indicated PBOX drugs, each at a final concentration of  $10 \mu\text{M}$  or (B) a range of concentrations of PBOX-6. The control wells in each case contained 0.5% (v/v) ethanol. After 16h the percent apoptosis and necrosis was determined by cytopinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the means  $\pm$  SEM for three separate experiments.



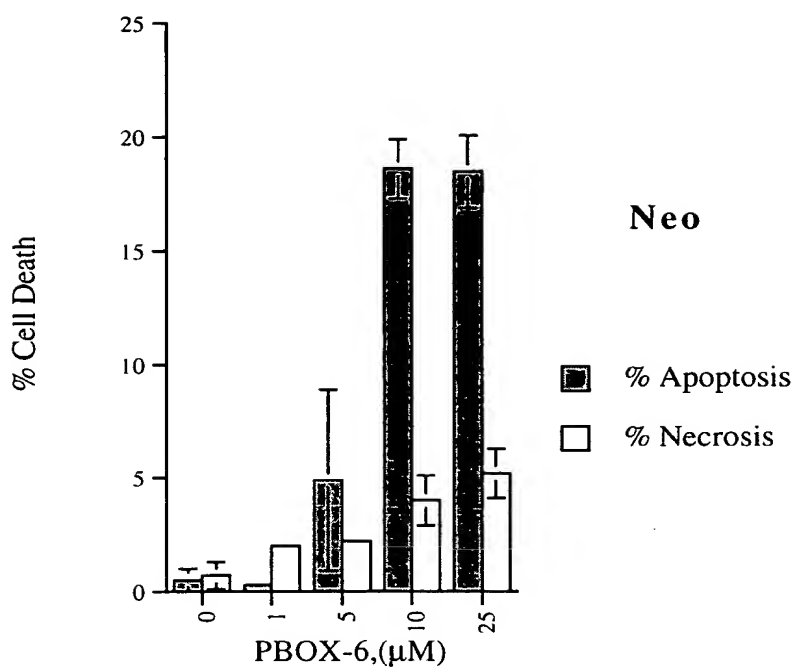
**Fig. 9. Lack of involvement of NFκB in pyrrolobenzoxazepine-induced apoptosis.**

(A) HL-60 cells were seeded at a density of  $3 \times 10^5$  cells/ml, and were pre-treated with TNFα (10ng/ml) for 1h followed by treatment with PBOX-6 for a further 16 h. The control wells in each case contained 0.5% (v/v) ethanol. Values represent the means  $\pm$  SEM of three separate experiments. (B) Same as in (A) but with Hut-78 cells incubated with either one of the indicated PBOX drugs, each at a final concentration of 10μM. Nuclear extracts (2μg) were prepared from (C) HL 60 cells treated either with control (0.5% (v/v) ethanol), TNFα (10ng/ml) or PBOX-6 (10μM) for 16 hours or (D) Same as in (C) but with Hut 78 cells. NFκB activity was then measured by EMSA described in the Methods section. The arrowhead represents NFκB-DNA. Results are representative of at least two separate experiments.



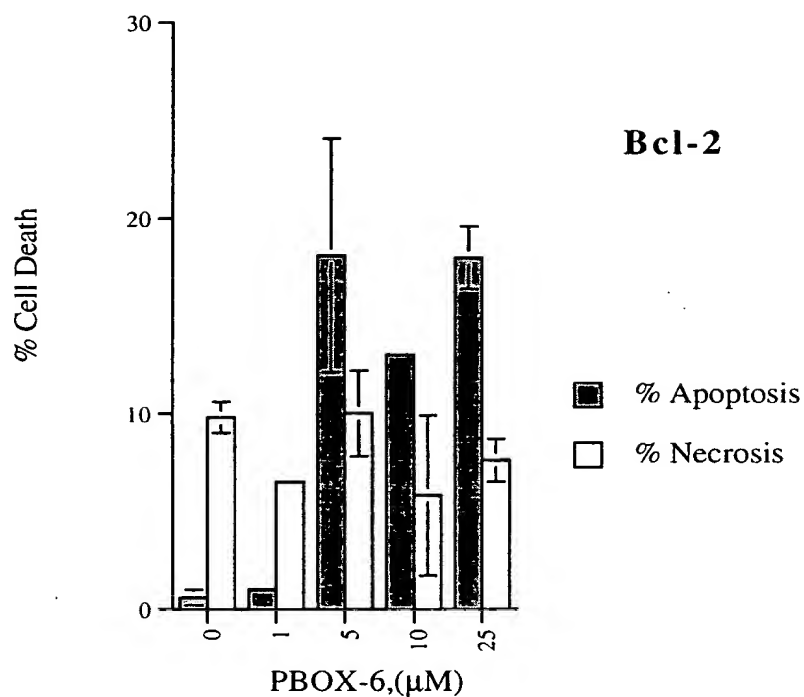
**Fig. 10. PBOX-6 induces transient activation of JNK in HL-60 cells.**

HL-60 cells were treated with either control (ethanol) or PBOX-6 for 1, 2 or 4 hr. A positive control for JNK activation was also set up by exposing the cells to UV light for 2 min followed by a 1 hr incubation at 37°C. Cytosolic extracts were then prepared and samples (30 µg of protein) were resolved by SDS-PAGE and probed for activated phosphorylated JNK. The two bands refer to the two JNK isoforms present (JNK 1 and JNK2, 45kDa and 54kDa respectively). Results are representative of at least two separate experiments.



**Fig. 11. PBOX-6 induces apoptosis in normal (neo) CEM cells.**

CEM cells were seeded at a density of  $3 \times 10^5$  cells/ml and were incubated with a range of concentrations of PBOX-6. The control wells in each case contained 0.5% (v/v) ethanol. After 16h the percent apoptosis and necrosis was determined by cytopinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the means  $\pm$  SEM for three separate experiments.

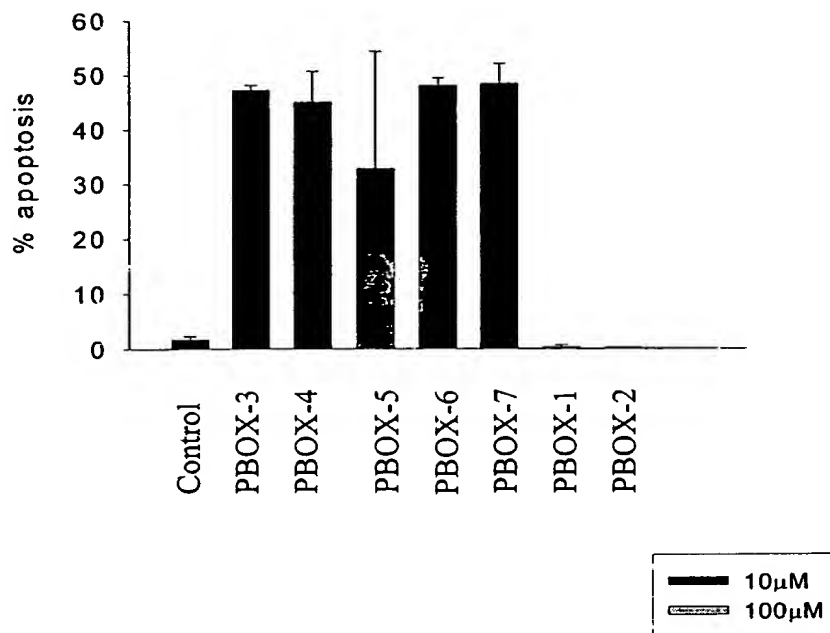


**Fig. 12. PBOX-6 induces apoptosis in Bcl-2 overexpressed CEM cells.**

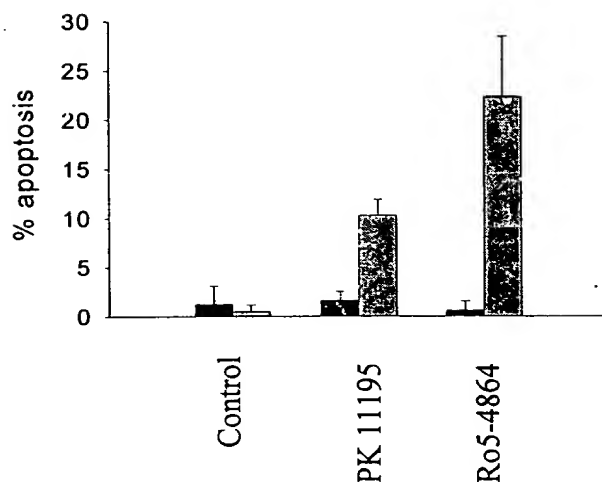
CEM cells, overexpressed with Bcl-2, were seeded at a density of  $3 \times 10^5$  cells/ml and were incubated with a range of concentrations of PBOX-6. The control wells in each case contained 0.5% (v/v) ethanol. After 16h the percent apoptosis and necrosis was determined by cytopinning and staining the cells using the RapiDiff kit as described in the Methods section. Values represent the means  $\pm$  SEM for three separate experiments.



A



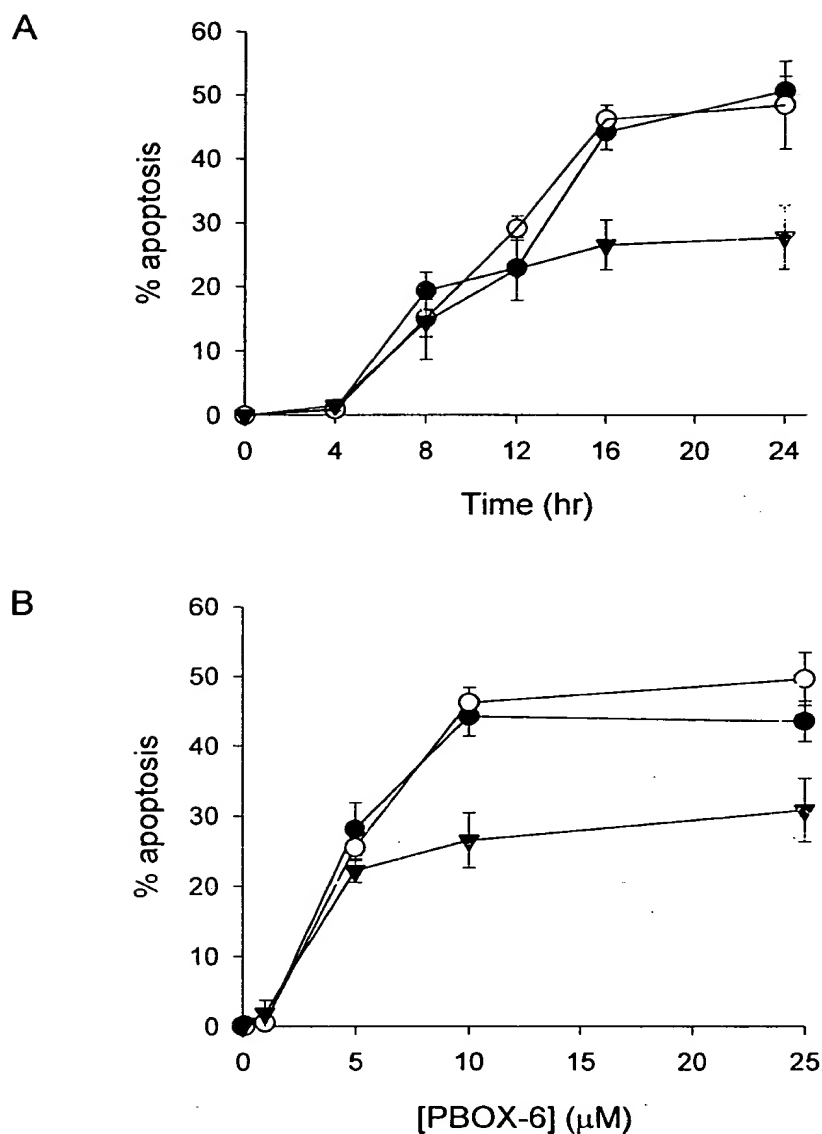
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**Fig. 13 Some pyrrolo-1,5-benzoxazepines and other PBR ligands induce apoptosis in K562 cells.**

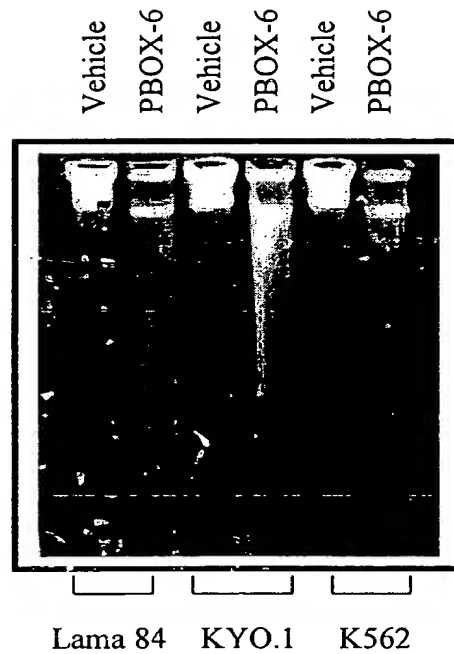
K562 cells were seeded at  $3 \times 10^5$  cells per ml and treated with (A) the indicated PBOX compound (10µM) for 16 hours or (B), PK 11195 or Ro5-4864 at either 10µM or 100µM. In each case a control was set up which contained 1% ethanol (v/v). Percent apoptosis was determined by cytopinning an aliquot (200µl) onto a glass slide and staining the cells using the RapiDiff kit. Values represent the mean  $\pm$  the range of 2 separate experiments.

● K562  
○ KYO.1  
▼ Lama 84



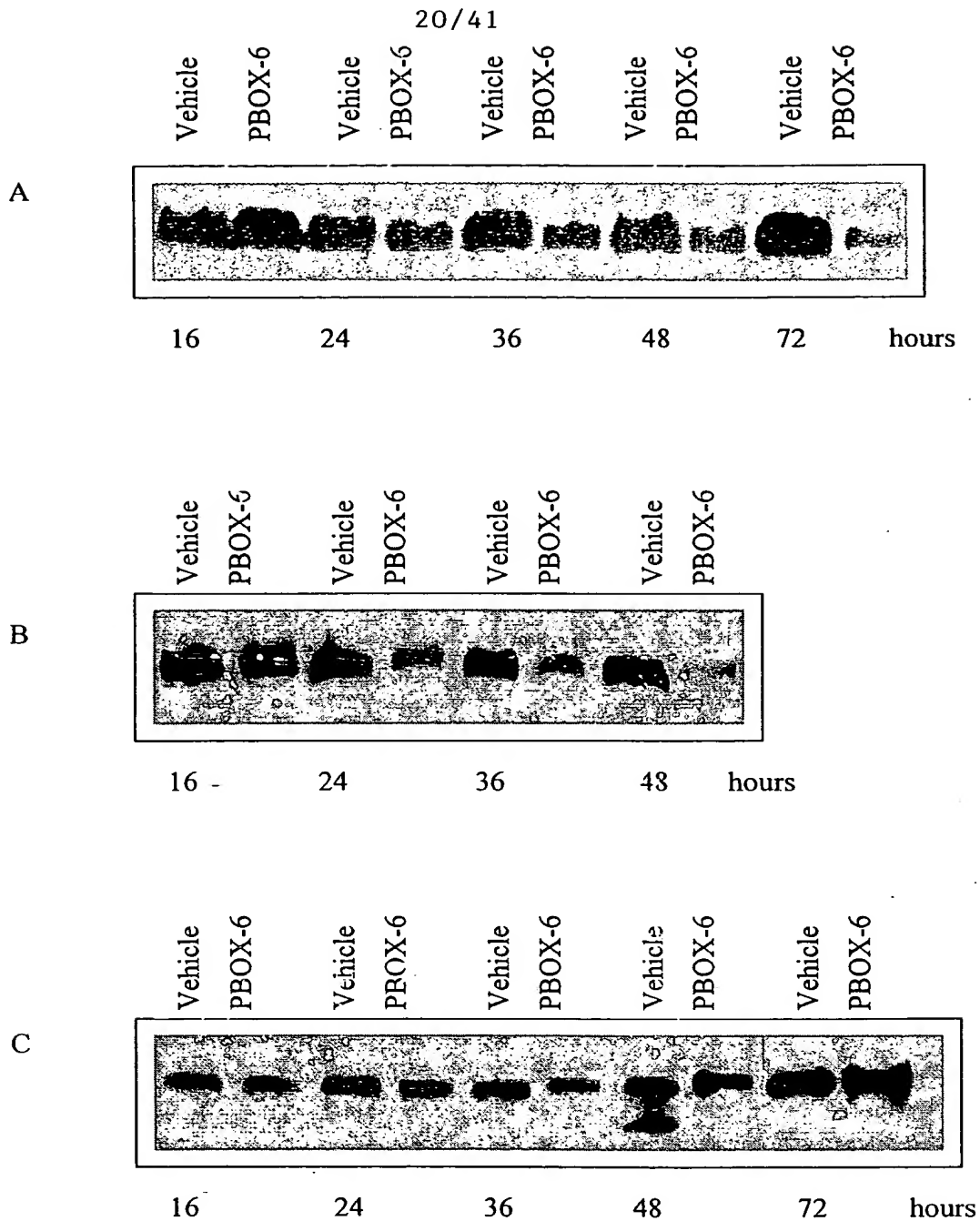
**Fig. 14 PBOX-6 induced apoptosis in CML cells is time and dose-dependent.**

CML cells were seeded at  $3 \times 10^5$  cells per ml and were treated with (A) PBOX-6 ( $10 \mu\text{M}$ ) for a period of 4, 8, 12, 16 and 24 hours or (B) a range (0- $25 \mu\text{M}$ ) concentrations of PBOX-6 for 16 hours. The percent apoptosis was determined by cytospinning and staining cells with the RapiDiff kit. Values represent the mean  $\pm$  SEM of 3 separate experiments.



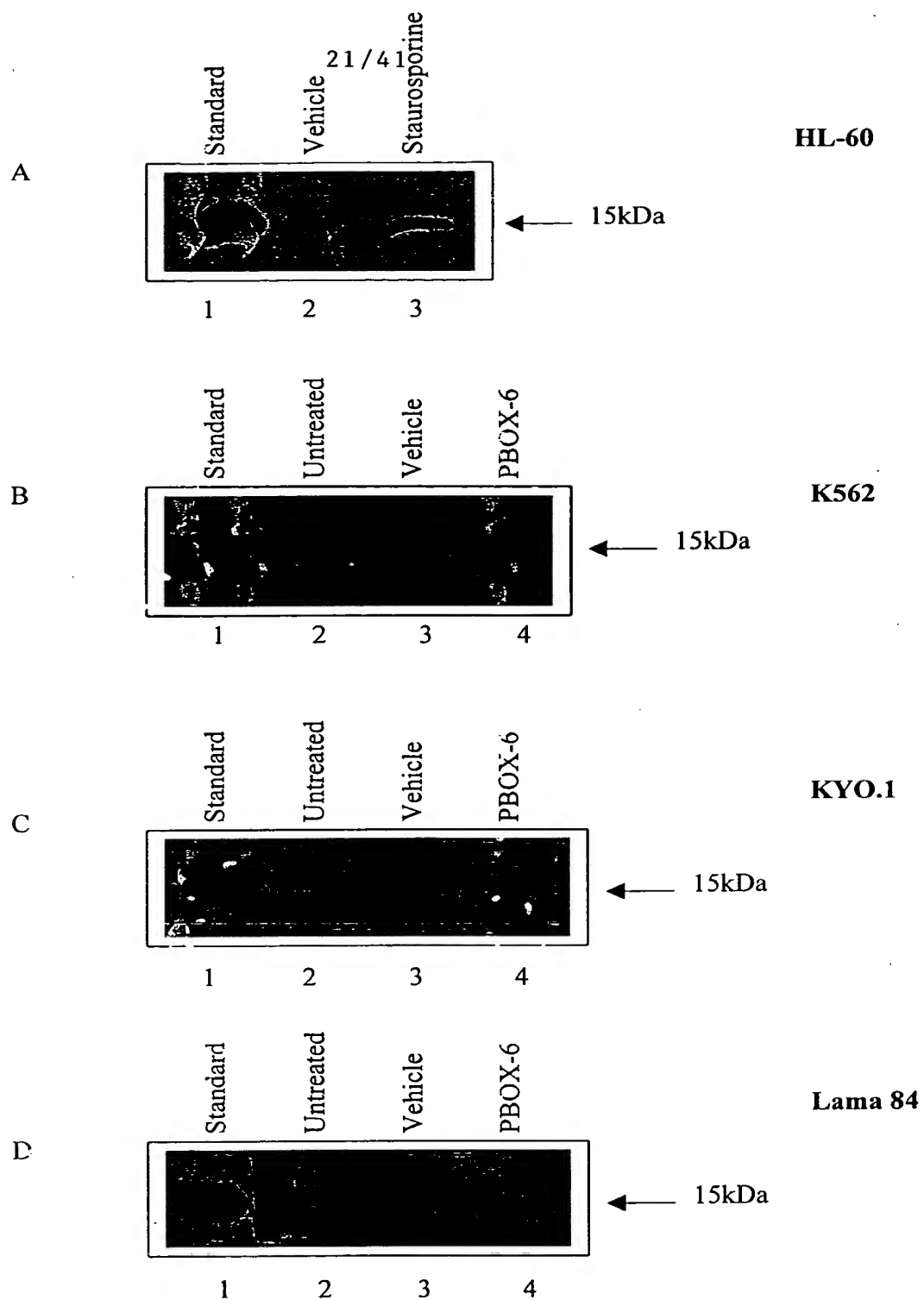
**Fig. 15 PBOX-6 induces internucleosomal DNA fragmentation in CML cells.**

Cells ( $1 \times 10^7$ ) were treated with either vehicle (1% ethanol) or PBOX-6 ( $10 \mu\text{M}$ ) for 48 hours and DNA ( $45 \mu\text{l}$ ) was resolved on an agarose gel (1.5%). DNA ladders were visible under UV light. Results are representative of at least 2 separate experiments.



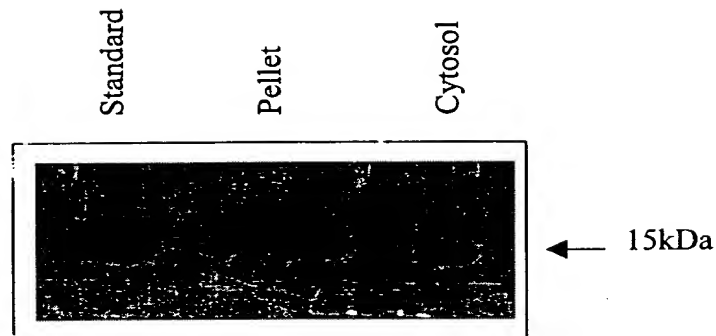
**Fig. 16 Downregulation of BCR-abl in K562 and KYO.1, but not in Lama 84 cells in response to PBOX-6 treatment.**

Cytosolic extracts were prepared from (A) K562 (B) KYO.1 and (C) Lama 84 cells following treatment with either vehicle (1% ethanol) or PBOX-6 (10 $\mu$ M) for 16, 24, 36, 48 and 72 hours. Protein (40 $\mu$ g) was resolved by SDS-PAGE and probed with anti-c-abl antibody. Results are representative of at least two separate experiments.



**Fig. 17 Cytochrome C western blotting**

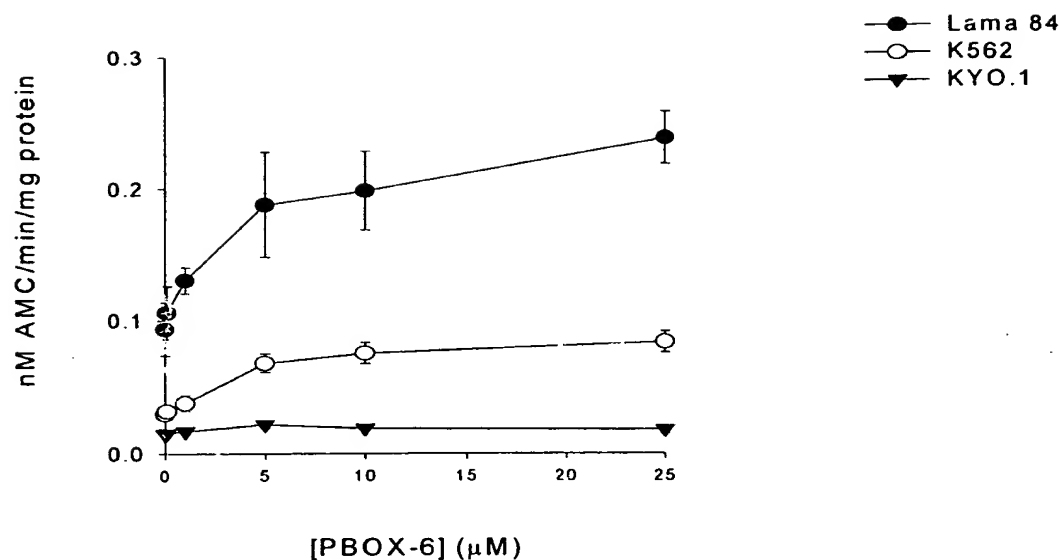
Cytosolic extracts were prepared from HL-60 cells (A) which were treated with either (lane 2-3), vehicle (0.1% DMSO) or staurosporine (1 $\mu$ M) for 6 hours. K562 (B), KYO.1 (C) and Lama 84 cells (D) were treated with either (lane 2-4) control (untreated), vehicle (1% ethanol) or PBOX-6 (10 $\mu$ M) for 16 hours. Protein (30 $\mu$ g) was resolved by SDS-PAGE and probed for cytochrome C. Horse Cytochrome C was used as a standard in each case (lane 1). Results are representative of at least 2 separate experiments.



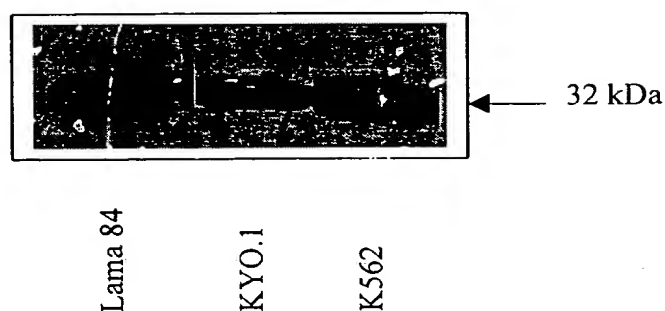
**Fig. 18 Cytochrome C is not all non-specifically released from Mitochondria during sample preparation.**

Cytosolic extracts from untreated K562 cells were prepared and the remaining pellet was solubilised in 100 $\mu$ l of buffer and incubated on ice for 30 min. Samples were centrifuged and the resulting superantant was removed. Protein (50 $\mu$ g) from the solubilised pellet (lane 2) and from the cytosol (lane 3) were resolved by SDS-PAGE and probed with anti-cytochrome C. Horse cytochrome C was used as a standard (lane 1).

A



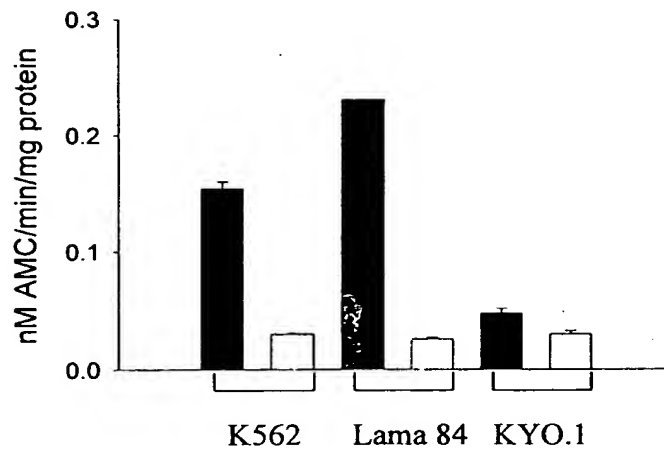
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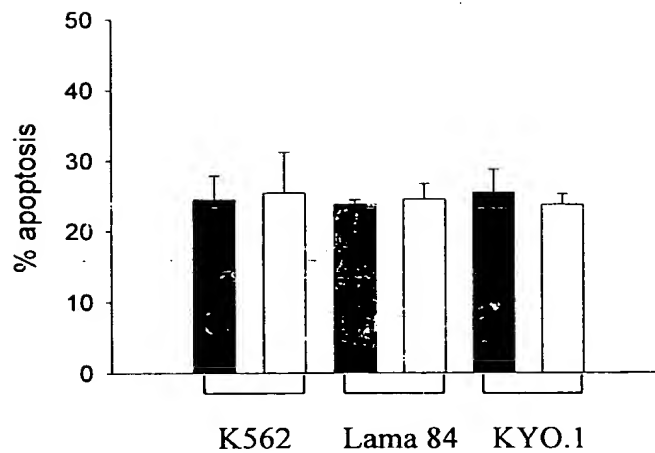
**Fig. 19 Activation of caspase 3 in K562 and lama 84 but not KYO.1 cells in response to PBOX-6 treatment.**

In (A) CML cells were seeded at  $5 \times 10^6$  cell per sample and treated with a range (0-25  $\mu$ M) of PBOX-6 concentrations for 16 hours. Cytosolic extracts were prepared and enzyme extracts (100  $\mu$ g) were incubated with substrate (20  $\mu$ M) in a total volume of 3 ml. All values represent the mean  $\pm$  SEM of three separate experiments. In (B) cytosolic extracts from  $6 \times 10^6$  untreated CML cells were prepared and protein (45  $\mu$ g) was resolved by SDS-PAGE before probing with anti-caspase 3 antibody. Results are representative of 2 separate experiments

A



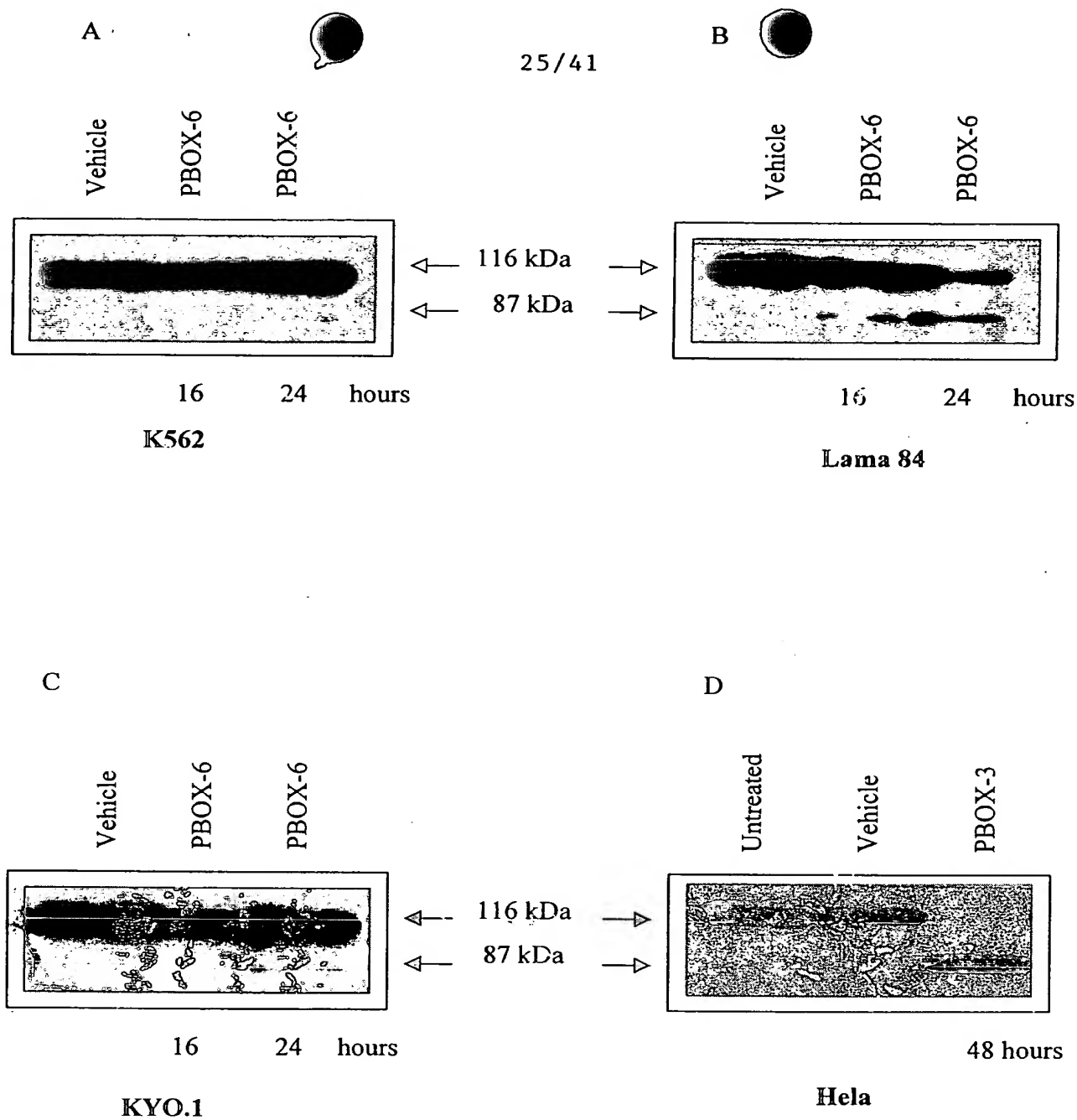
B



**Fig. 20 Caspase 3 inhibitor, z-DEVD-fmk, fails to protect against PBOX-6 induced apoptosis in CML cells.**

CML cells were seeded at (A)  $5 \times 10^6$  cells per ml and treated with PBOX-6 (10 μM) for 16 hours. Cytosolic extracts were prepared and enzyme extracts (100 μg) were incubated with or without z-DEVD-fmk for 1 hour at room temperature prior to the addition of caspase 3 substrate (20 μM). CML cells were seeded at (B)  $3 \times 10^5$  cells per ml and pretreated with z-DEVD-fmk for 1 hour prior to treatment with PBOX-6 (10 μM) for a further 8 hours. Percent apoptosis was determined by RapiDiff staining. Values represent the mean  $\pm$  SEM of 3 separate experiments.

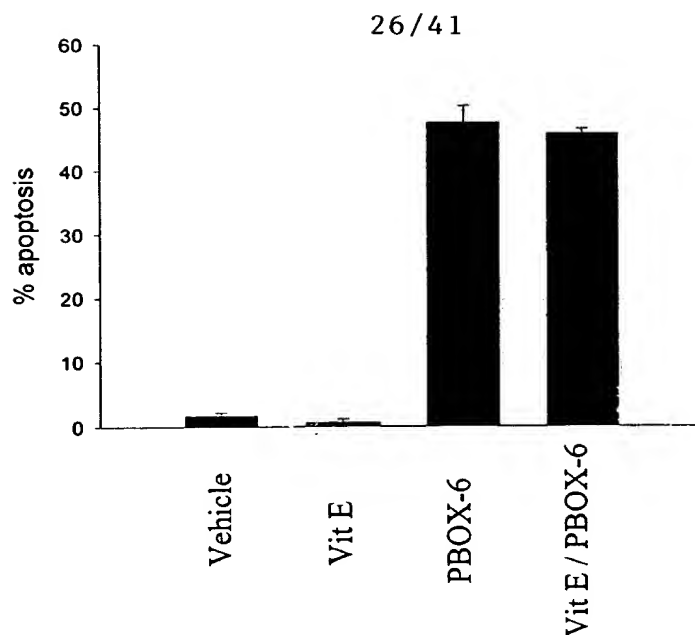




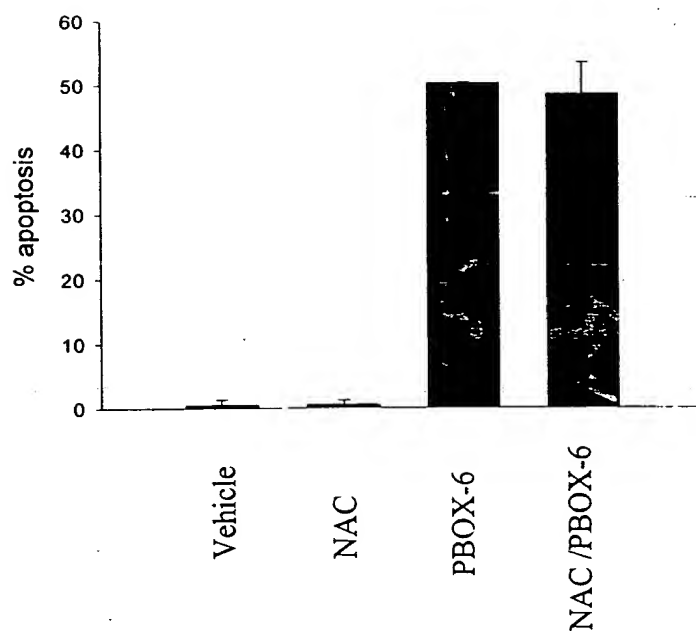
**Fig. 21 Induction of PARP cleavage in CML and Hela cells following treatment with PBOX-6 and PBOX-3.**

Whole cell extracts from K562 (A), Lama 84 (B), KYO.1 (C) and Hela cells (D) were prepared following treatment with either PBOX-6 (10 $\mu$ M) for 16 and 24 hours (A, B, and C) or PBOX-3 (10 $\mu$ M) for 48 hours (D). In each case a vehicle treated control was set up containing 1% ethanol. Samples were resolved by SDS-PAGE and probed with anti-PARP antibody. Results are representative of at least 2 experiments.

A

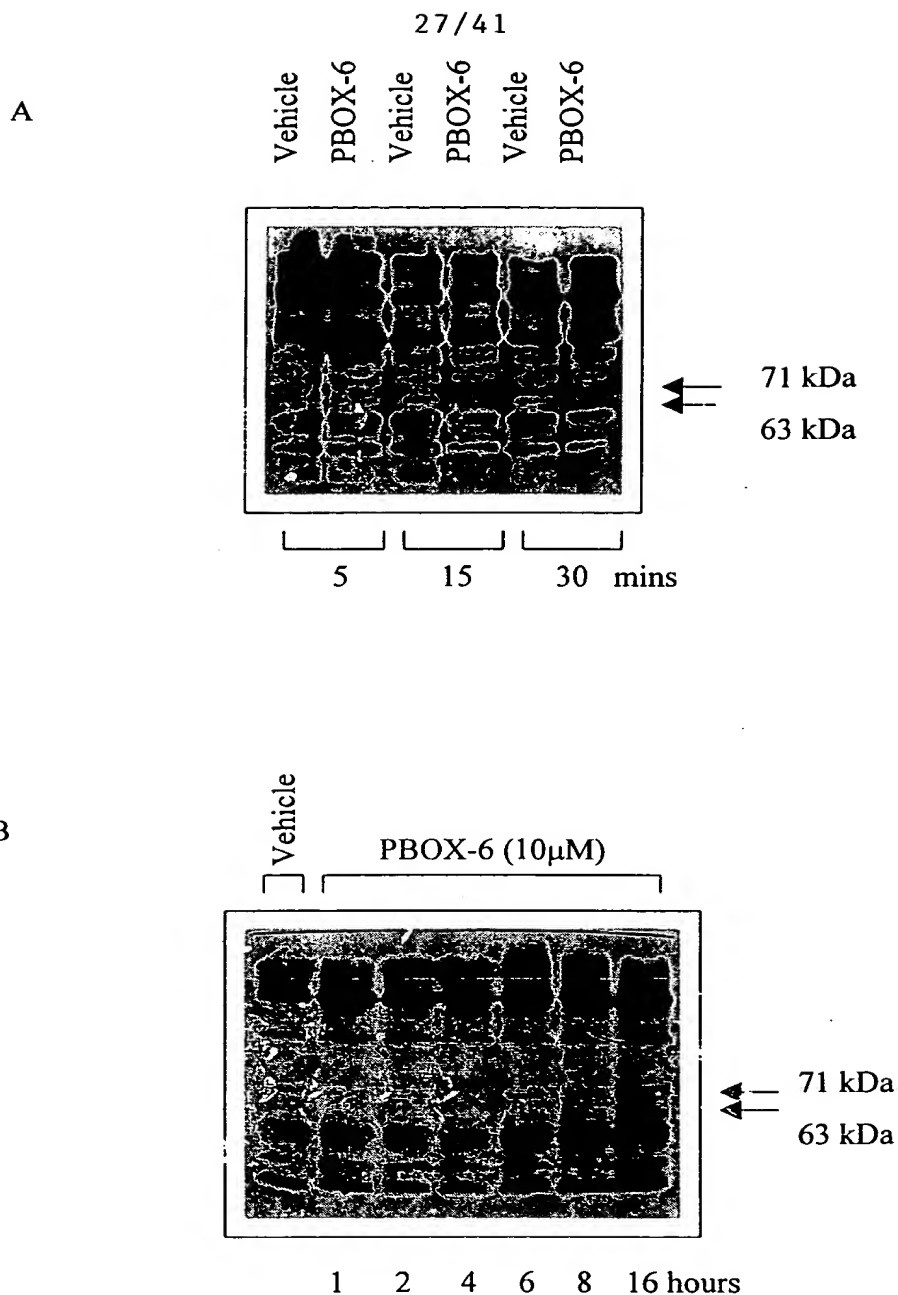


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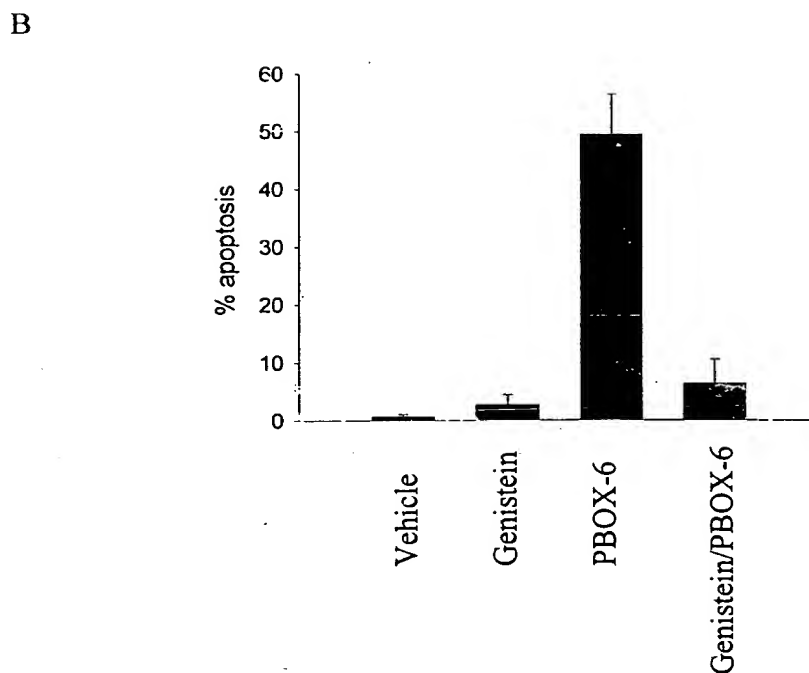
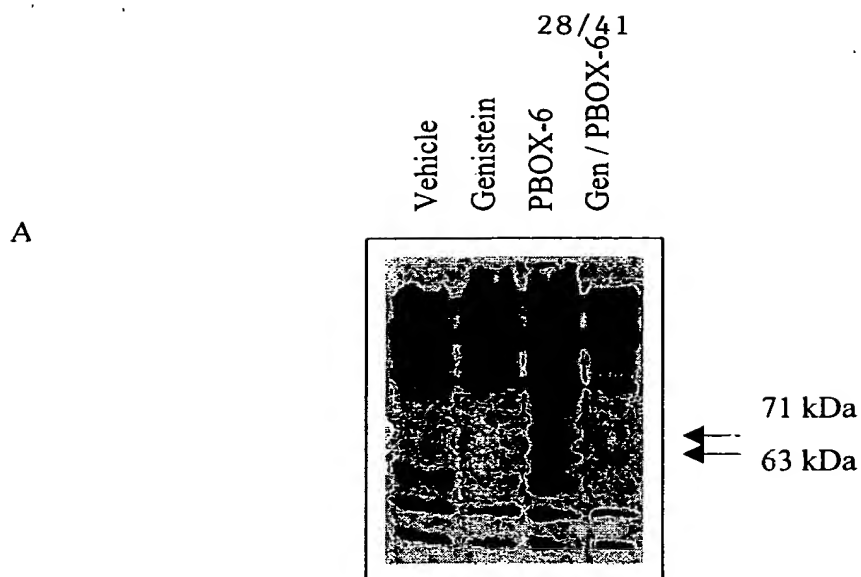


**Fig. 22 Antioxidants fail to protect against PBOX-6 induced apoptosis in K562 cells.**

K562 cells were seeded at  $3 \times 10^5$  cells per ml and treated with (A) either vehicle (1% PBS, 0.1% ethanol), Vitamin E (100 $\mu$ M) for 40 hours, PBOX-6 (10 $\mu$ M) for 16 hours or a pretreatment of Vitamin E for 24 hours followed by PBOX-6 for a further 16 hours. In (B) cells were treated with either vehicle (25mM Tris, 0.1% ethanol), N-Acetylcysteine (NAC) (5mM) for 17 hour, or a pretreatment of NAC for 1 hour followed by PBOX-6 for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Results represent the mean  $\pm$  SEM of 3 separate experiments.

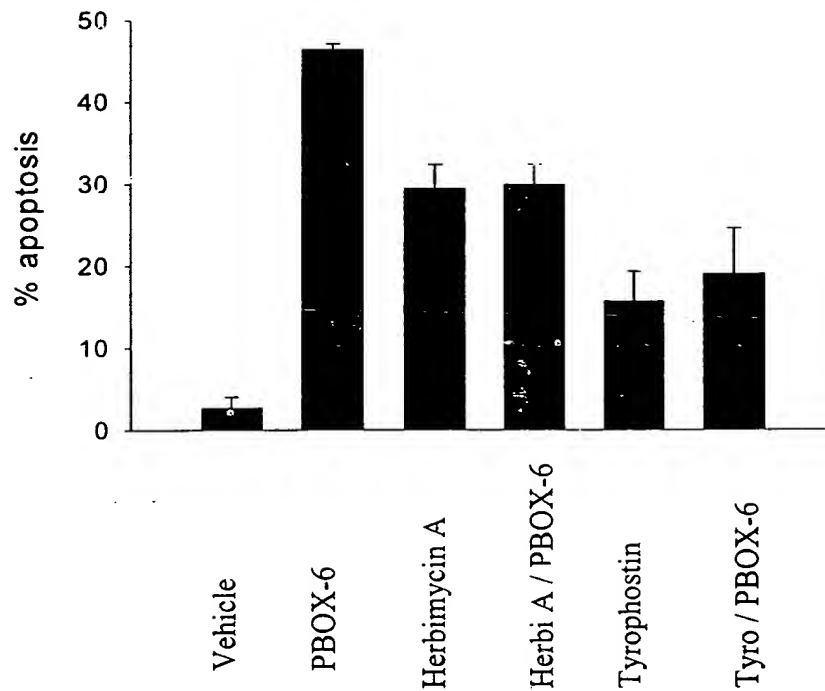


**Fig. 23 PBOX-6 alters the tyrosine phosphorylation status of proteins in K562 cells.** K562 cells ( $5 \times 10^6$ ) were treated with vehicle (1% ethanol) or PBOX-6 (10 $\mu$ M) for either (A) 5, 15 and 30 min or (B) 1, 2, 4, 6, 8, and 16 hours. Cytosolic extracts were prepared and protein (40 $\mu$ g) was resolved by SDS-PAGE and probed with anti-phosphotyrosine antibody. Results are representative of at least 2 experiments.



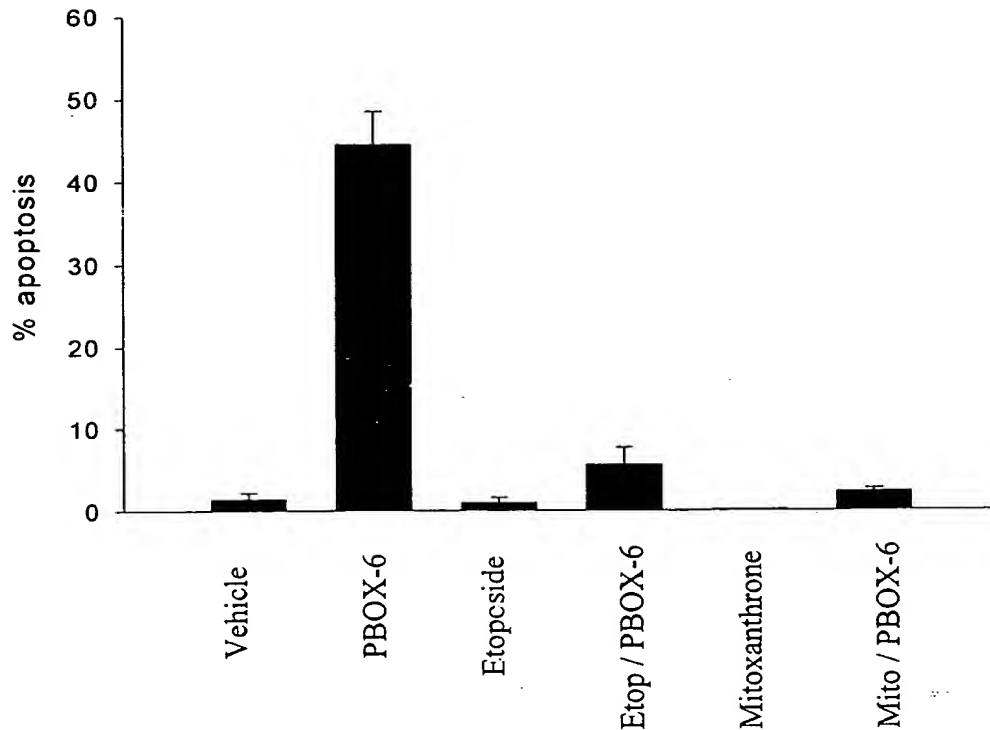
**Fig. 24 Pretreatment of K562 cells with the tyrosine kinase inhibitor, Genistein, prevents protein tyrosine phosphorylation and inhibits apoptosis induced by PBOX-6.**

K562 cells were seeded at (A)  $5 \times 10^6$  cells per sample or (B)  $3 \times 10^5$  cells per ml and pretreated with genistein ( $100 \mu\text{M}$ ) for 1 hour prior to treatment with PBOX-6 ( $10 \mu\text{M}$ ) for a further 16 hours. In (A) cytosolic extracts were prepared and protein ( $40 \mu\text{g}$ ) was resolved by SDS-PAGE and probed with anti-phosphotyrosine antibody. Results are representative of at least 3 experiments. In (B) percent apoptosis was determined by RapiDiff staining. Results represent the mean  $\pm$  SEM of 3 separate experiments.



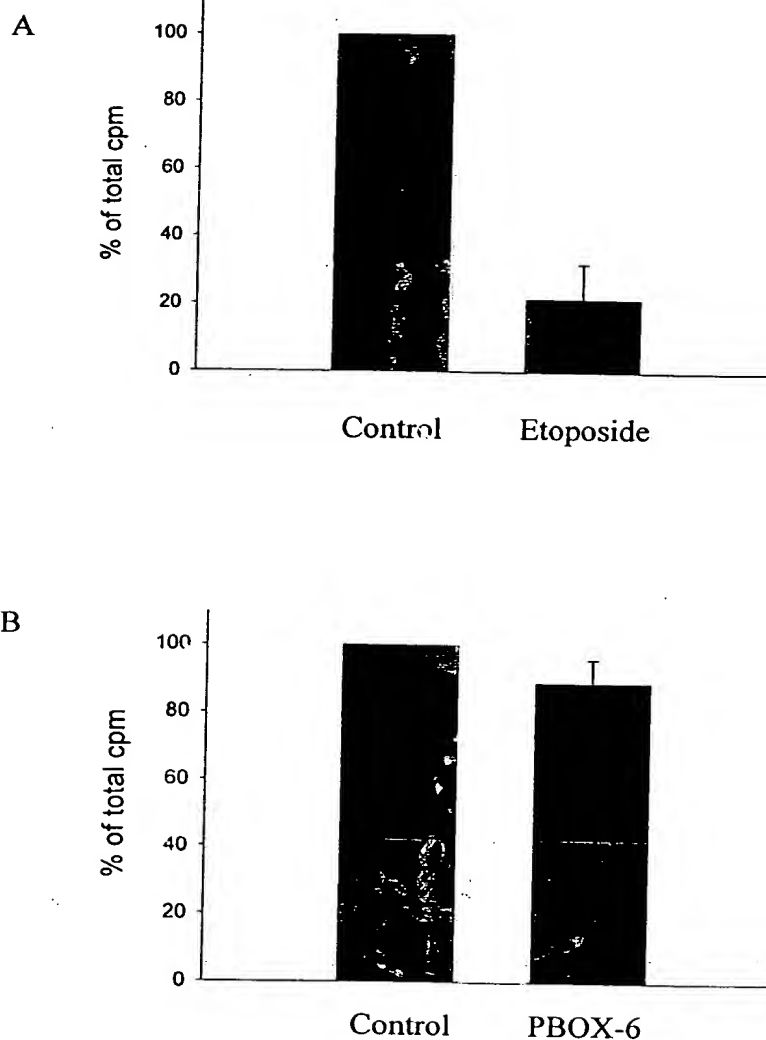
**Fig. 25 Herbimycin A and Tyrophostin reduce, but do not completely inhibit, PBOX-6 induced apoptosis in K562 cells.**

Cells were seeded at  $3 \times 10^5$  cells per ml and were pretreated with either Herbimycin A ( $5 \mu\text{M}$ ) or Tyrophostin ( $200 \mu\text{M}$ ) for 1 hour prior to treatment with PBOX-6 ( $10 \mu\text{M}$ ) for a further 16 hours. Percent apoptosis was determined by cytopinning the cells onto a slide and staining with the RapiDiff kit. Results represent the mean  $\pm$  SEM of 3 separate experiments.



**Fig. 26 Etoposide and mitoxanthrone pretreatment of K562 cells protects against PBOX-6 induced apoptosis.**

K562 cells were seeded at a density of  $3 \times 10^5$  cells per ml and pretreated with either etoposide ( $50 \mu\text{M}$ ) or mitoxanthrone ( $500 \text{nM}$ ), for 1 hour, before treating with PBOX-6 ( $10 \mu\text{M}$ ) for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Values represent the mean  $\pm$  SEM of 3 separate experiments.



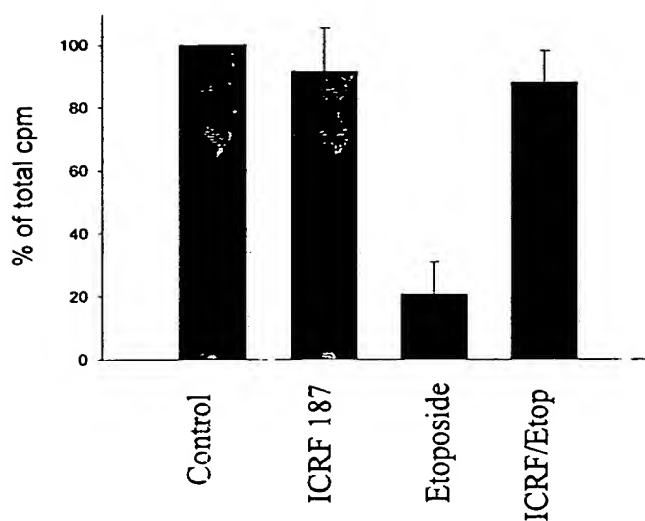
**Fig. 27 PBOX-6 does not induce DNA strand breaks in K562 cells.**

Jurkat cells (A) were treated with vehicle (0.1% ethanol:DMSO (1:1)) or etoposide (2.5 $\mu$ M) for 1 hour and K562 cells (B) were treated with vehicle (1% ethanol) or PBOX-6 (10 $\mu$ M) for 3 hours. Cells were lysed on filters and eluted overnight at 0.05ml/min using an alkaline solution. Results represent the mean  $\pm$  SEM of 3 separate experiments. Student's t-test was carried out using the Instat programme.

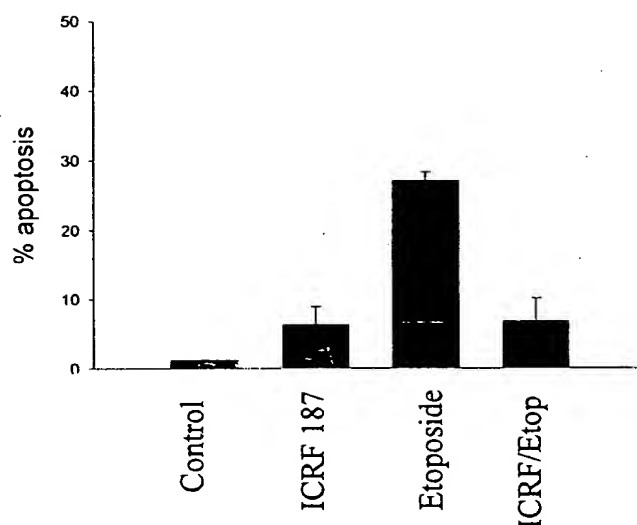
$p < 0.01$  ; etoposide when compared to control

$p = 0.2148$  ; PBOX-6 when compared to control

A



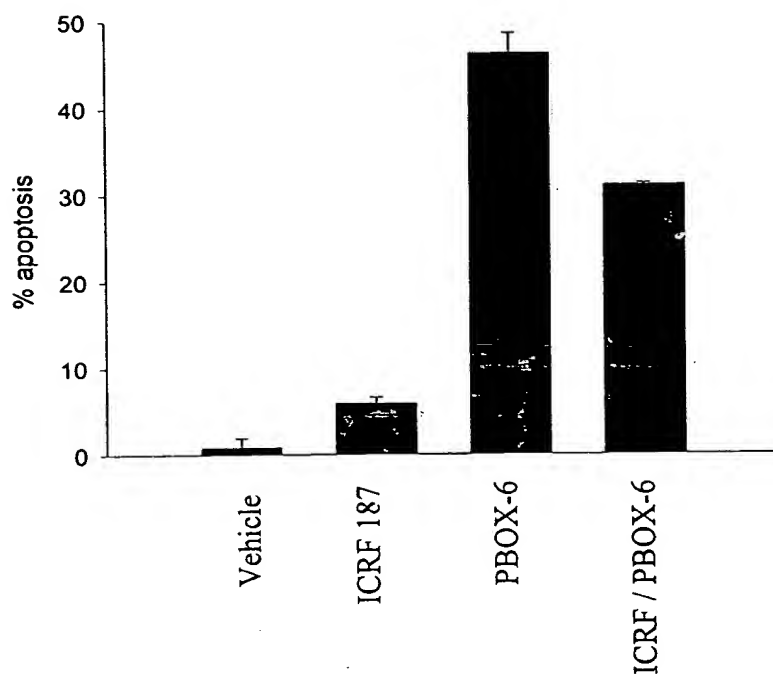
B



**Fig. 28 Pretreatment of Jurkat cells with ICRF 187 inhibits etoposide induced DNA strand breaks and protects against apoptosis.**

Jurkat cells were either (A) treated with vehicle (0.1% ethanol:DMSO (1:1)), ICRF 187 (200 $\mu$ M) for 1 hour, etoposide (2.5 $\mu$ M) for 1 hour or a pretreatment of ICRF 187 for 1 hour prior to treatment with etoposide for a further hour. Cells were lysed onto filters and eluted overnight. In (B) Jurkat cells were seeded at  $3 \times 10^5$  cells/ml and treated with either vehicle (0.1% ethanol:DMSO (1:1)), ICRF 187 (200 $\mu$ M) for 17 hours, etoposide (2.5 $\mu$ M) for 16 hours or a pretreatment of ICRF 187 for 1 hour followed by etoposide for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Values represent the mean  $\pm$  range of 2 separate experiments.



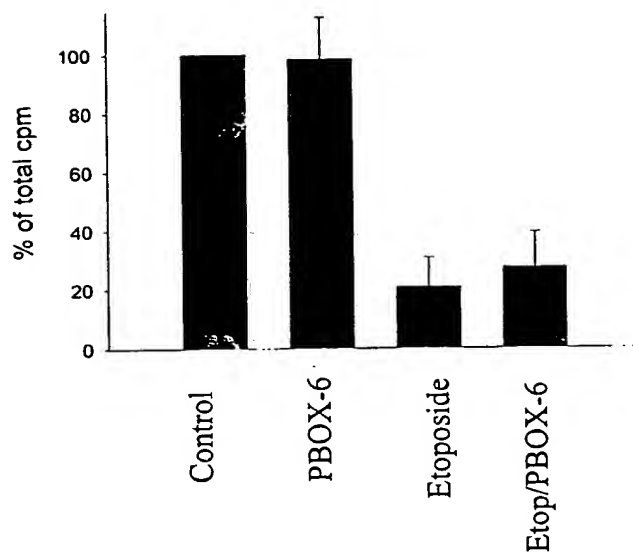


**Fig. 29 Pretreatment of K562 cells with ICRF 187 reduces the level of apoptosis induced by PBOX-6.**

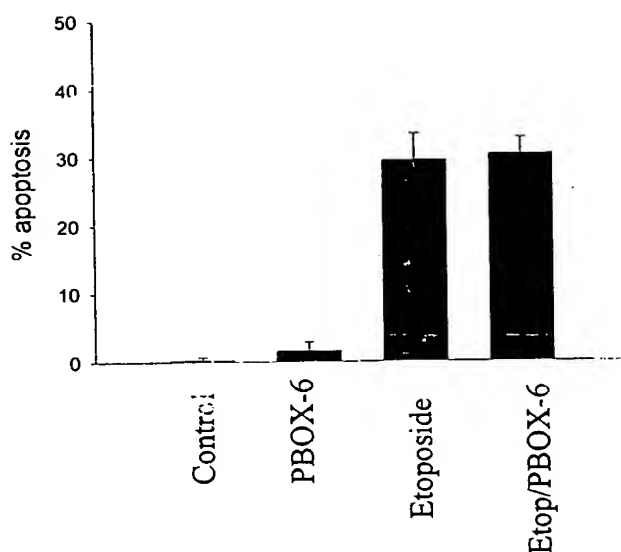
K562 cells were seeded at  $3 \times 10^5$  cells per ml and treated with either vehicle (1% ethanol), ICRF 187 (200 $\mu$ M) for 17 hours, PBOX-6 (10 $\mu$ M) for 16 hours or a pretreatment of ICRF 187 for 1 hour prior to treatment with PBOX-6 for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Results are representative of at least 2 separate experiments

34/41

A

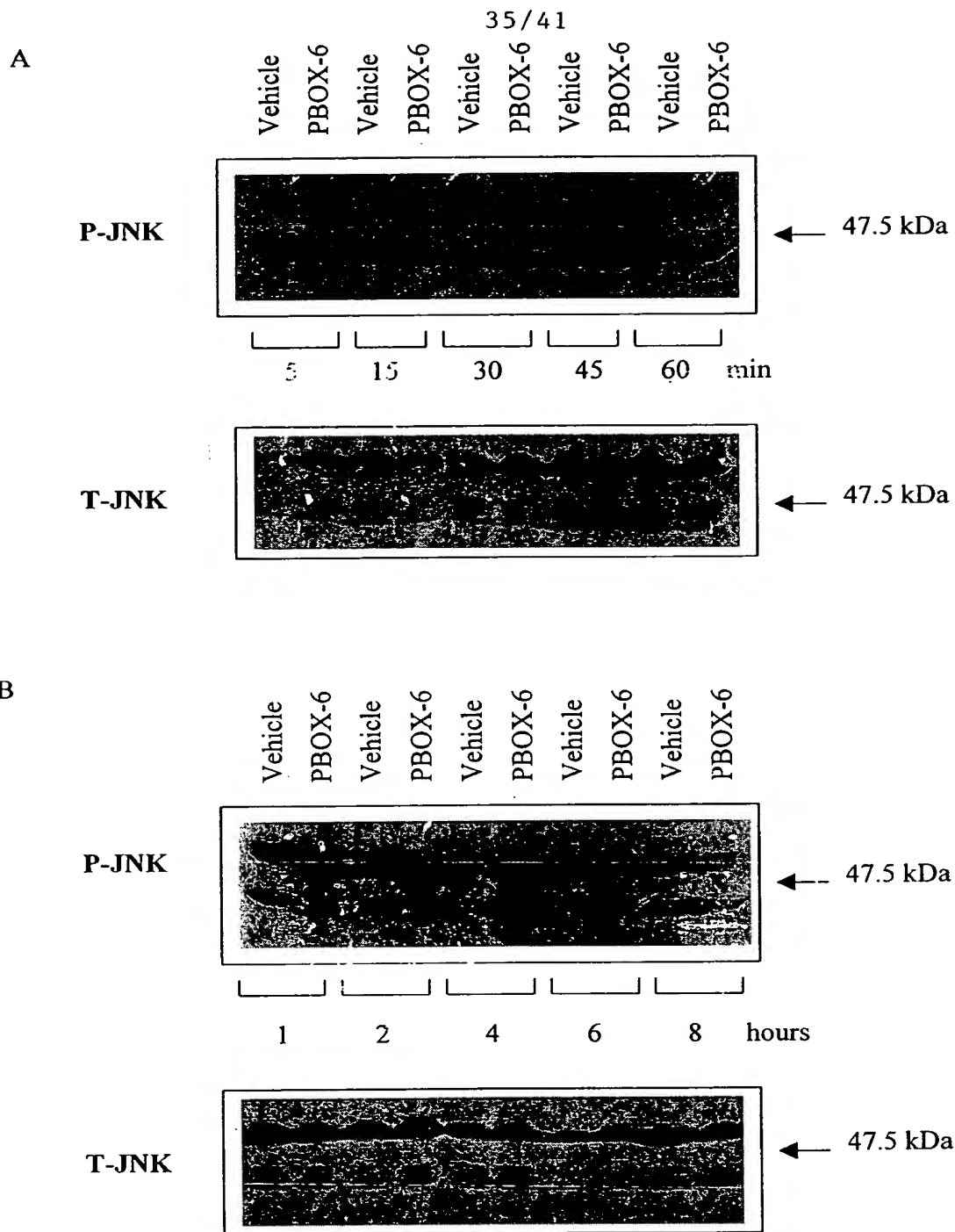


B



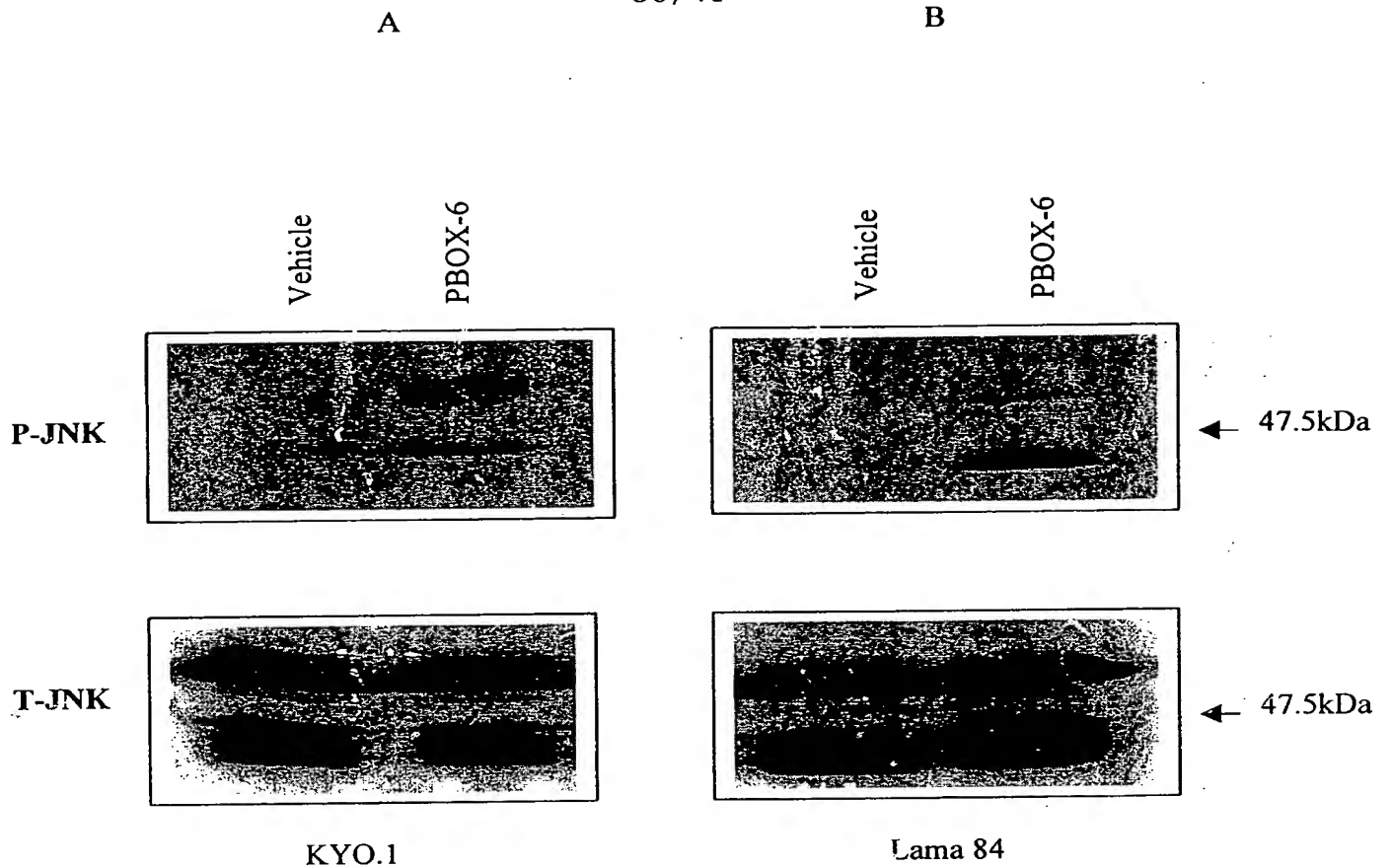
**Fig. 30 Pretreatment of Jurkat cells with PBOX-6 failed to protect against DNA strand breaks or apoptosis induced by etoposide.**

Jurkat cells were either set up as outlined for alkaline elution (A) or seeded at  $3 \times 10^5$  cells per ml (B) and treated with (A) vehicle (1% ethanol, 0.1% DMSO), PBOX-6 (10  $\mu$ M) for 3 hours, etoposide (2.5  $\mu$ M) for 1 hour or a pretreatment of PBOX-6 (0.5  $\mu$ M) for 1 hour followed by etoposide for a further hour. Cells were lysed onto filters and eluted overnight. In (B) cells were treated with vehicle (1% ethanol, 0.1% DMSO), PBOX-6 (0.5  $\mu$ M) for 17 hours, etoposide (2.5  $\mu$ M) for 16 hours or a pretreatment of PBOX-6 for 1 hour followed by etoposide for a further 16 hours. Percent apoptosis was determined by RapiDiff staining. Values represent the mean  $\pm$  SEM of 3 separate experiments.



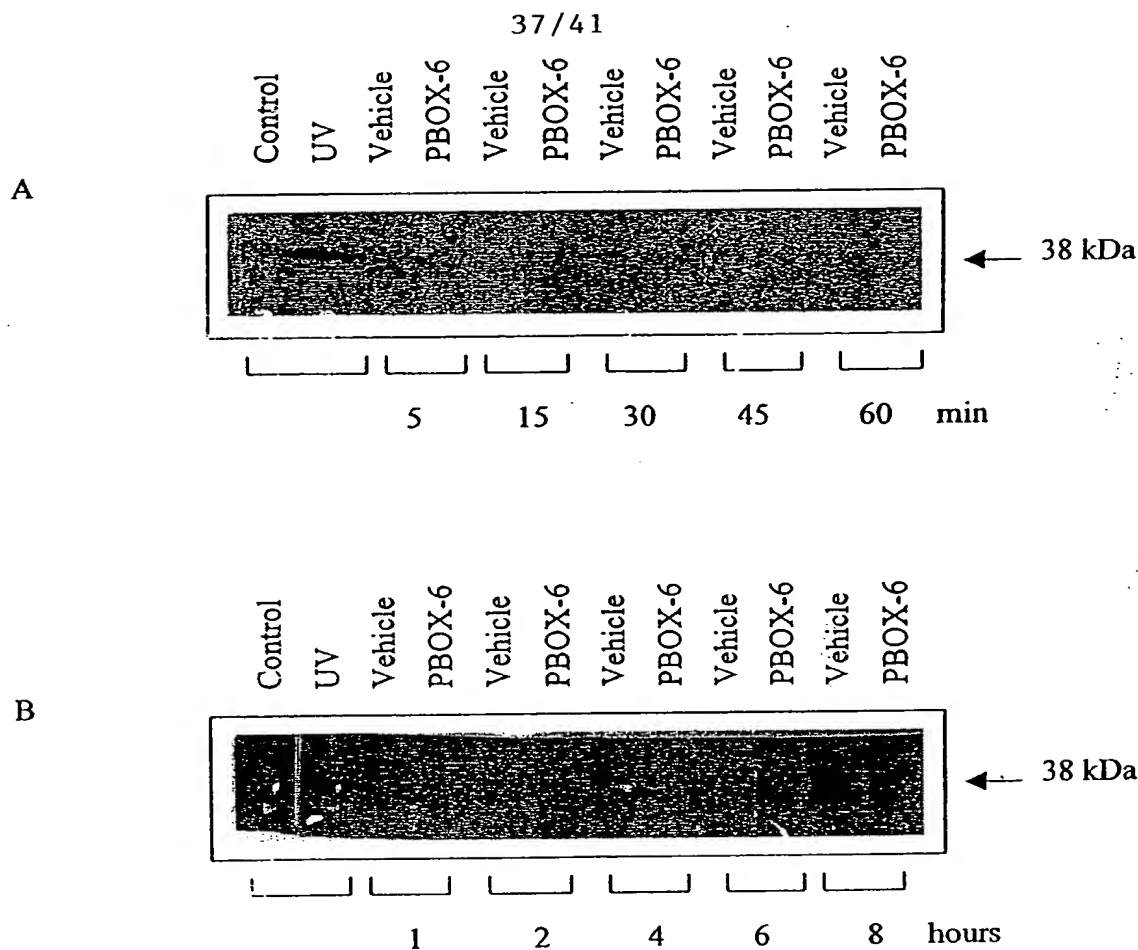
**Fig. 31 PBOX-6 induces transient activation of JNK in K562 cells.**

K562 cells were seeded at  $6 \times 10^6$  cells per sample and treated with either vehicle (1% ethanol) or PBOX-6 ( $10 \mu\text{M}$ ) for (A) 5, 15, 30, 45 and 60 min, or (B) 1, 2, 4, 6 and 8 hours. Whole cell extracts were prepared and protein ( $40 \mu\text{g}$ ) was resolved by SDS-PAGE. Blots were probed with anti-JNK phospho antibody and were then stripped and re-probed with anti-JNK total as a loading control. Results are representative of two separate experiments.



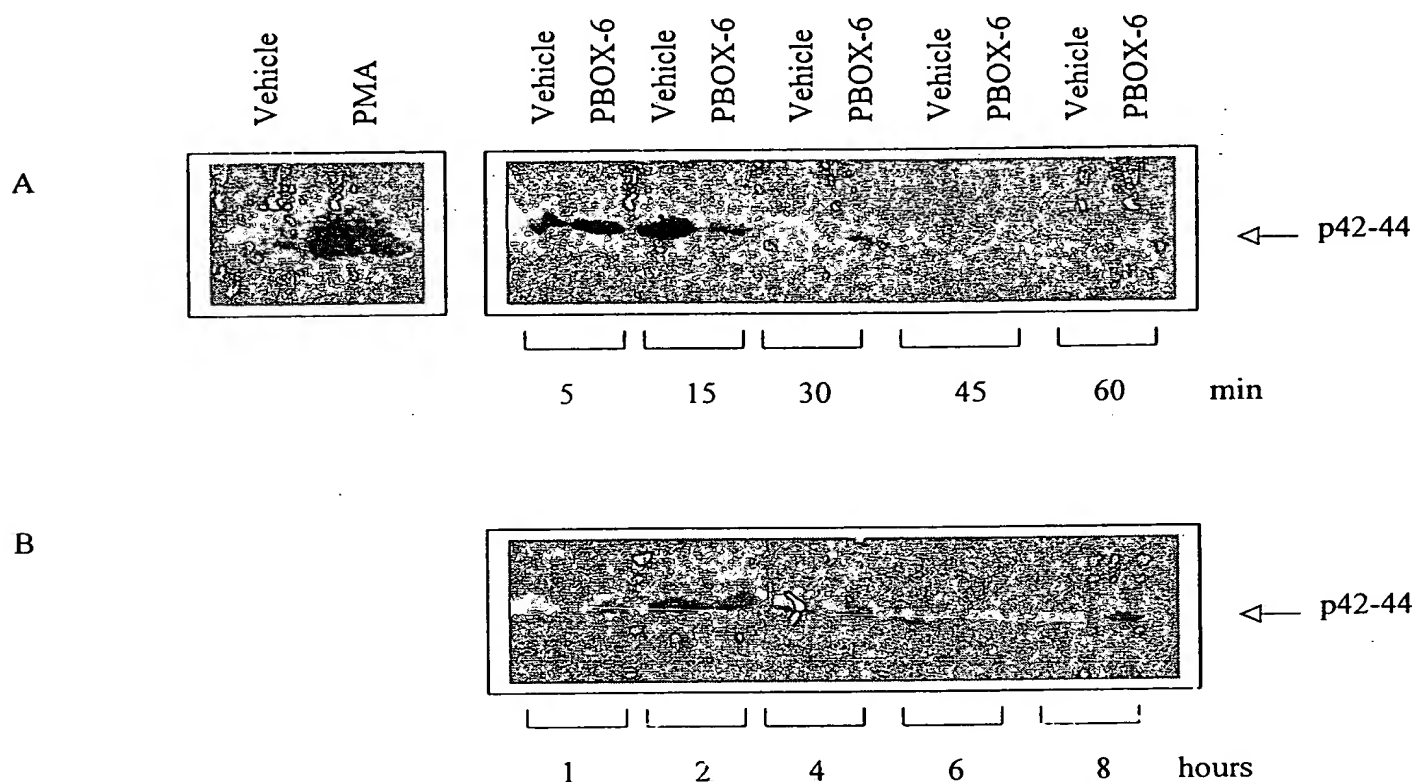
**Fig. 32 PBOX-6 induces activation of JNK in KYO.1 and Lama 84 cells.**

KYO.1 (A) and Lama 84 cells (B) were seeded at  $6 \times 10^6$  cells per sample and treated with either vehicle (1% ethanol) or PBOX-6 (10 $\mu$ M) for 45 minutes. Whole cell extracts were prepared and protein (50 $\mu$ g) was resolved by SDS-PAGE. Blots were incubated with anti-JNK phospho antibody and then stripped and re-probed with anti-JNK total antibody as a loading control. Results are representative of two separate experiments.



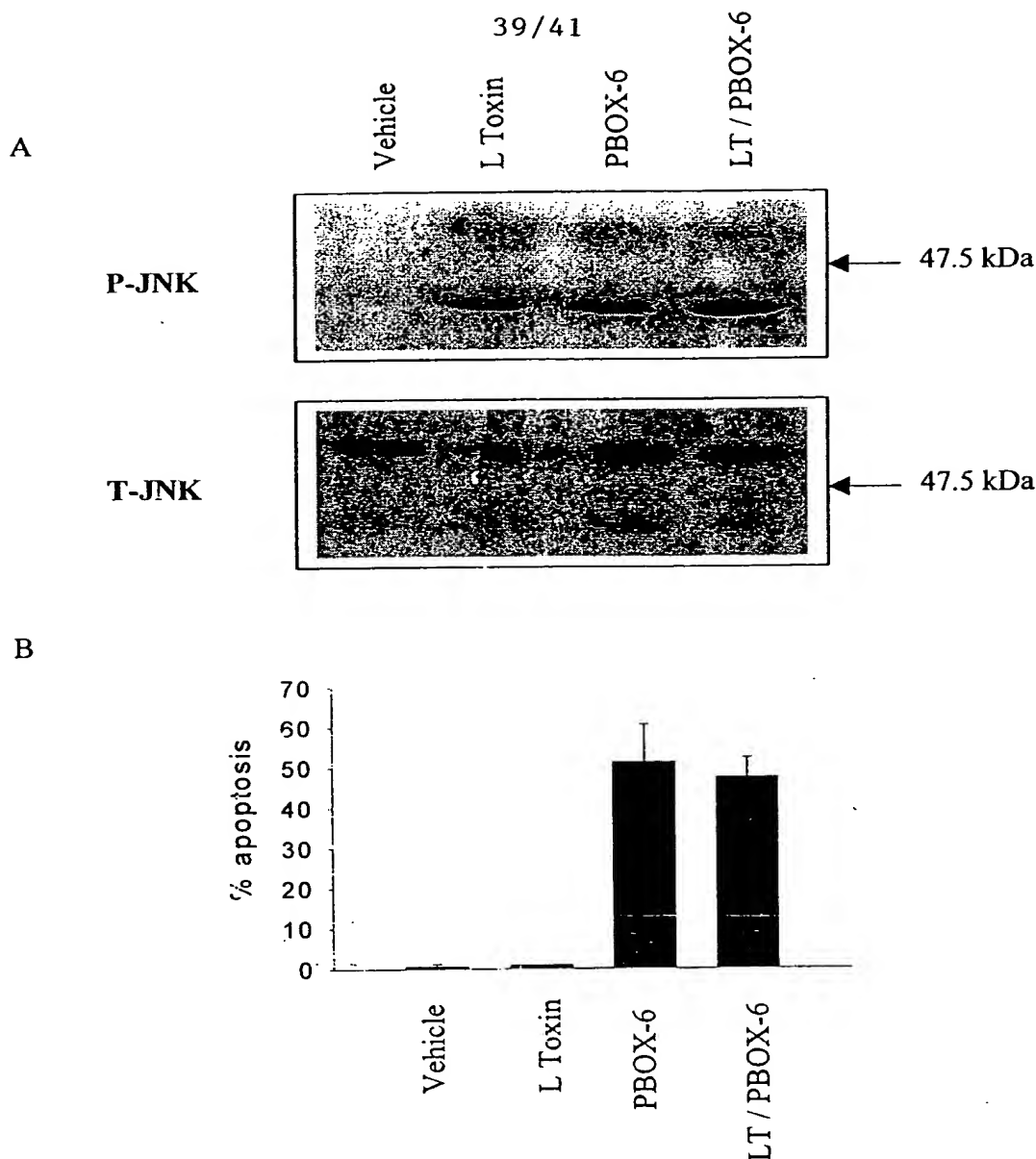
**Fig. 33 Lack of activation of p38 in K562 cells in response to PBOX-6.**

Cells were seeded at  $6 \times 10^6$  cells per sample. Jurkat cells (lanes 1-2) were UV irradiated for 2 min and K562 cells (lanes 3-12) were treated with vehicle (1% ethanol) or PBOX-6 ( $10 \mu\text{M}$ ) for either (A) 5, 15, 30, 45 and 60 min or (B) 1, 2, 4, 6 and 8 hours. Extracts were prepared and probed with anti-p38 antibody. Values represent the results of 2 separate experiments.



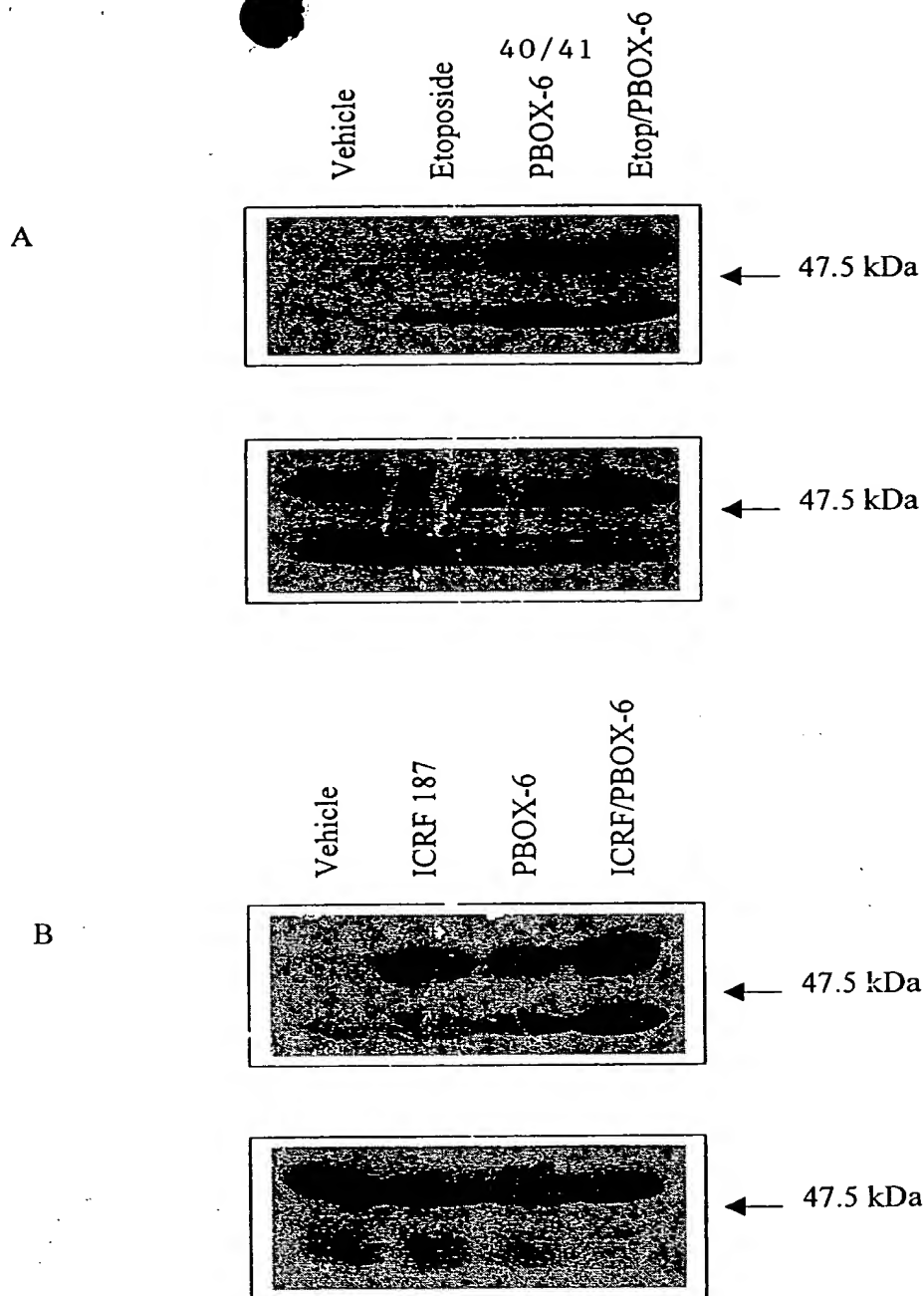
**Fig. 34 Lack of activation of p42-44 in K562 cells in response to PBOX-6.**

K562 cells were seeded at  $6 \times 10^6$  cells per sample and treated with either vehicle (1% ethanol) or PBOX-6 ( $10 \mu\text{M}$ ) for (A) 5, 15, 30, 45 and 60 min or (B) 1, 2, 4, 6 and 8 hours. Whole cell extracts were prepared as described in materials and methods. Protein ( $50 \mu\text{g}$ ) was resolved by SDS-PAGE and probed with anti-p42-44 phospho antibody. As a positive control, K562 cells were treated with PMA ( $100 \text{ nM}$ ) for 30 minutes. Results are representative of 2 separate experiments.



**Fig. 35 Pretreatment of K562 cells with an inhibitor of Rac 1, lethal toxin, failed to protect against PBOX-6 induced JNK activation and apoptosis.**

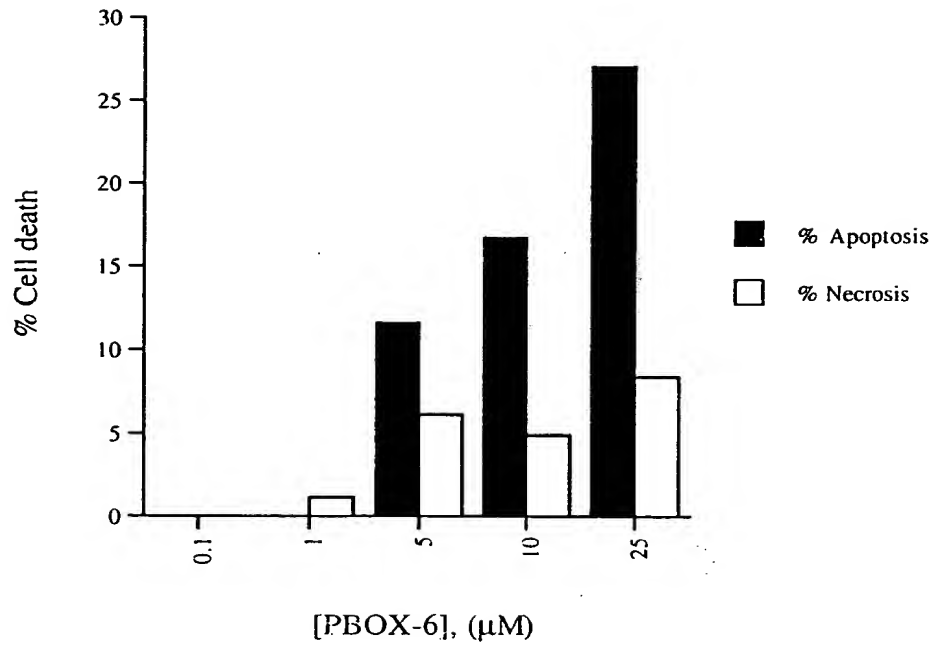
K562 cells were seeded at either (A)  $6 \times 10^6$  cells per sample or (B)  $3 \times 10^5$  cells per ml and pretreated with either (A) lethal toxin (500ng/ml) for 3 hours followed by PBOX-6 (10 $\mu$ M) for a further 45 mins. Protein (50 $\mu$ g) was resolved by SDS-PAGE and probed with anti-JNK phospho antibody. Blots were stripped and re-probed with anti-JNK total antibody as a loading control. In (B) cells were pretreated with lethal toxin (500ng/ml) for 1 hour prior to treatment with PBOX-6 (10 $\mu$ M) for a further 16 hours. Cells were spun onto a slide and percent apoptosis was determined using RapiDiff staining. Result are representative of 2 separate experiments.



**Fig. 36 Activation of JNK lies upstream of a requirement for Topo II in the pathway by which PBOX-6 induces apoptosis in K562 cells.**

K562 cells were seeded at  $6 \times 10^6$  cells per sample and pretreated with either (A) etoposide ( $50 \mu\text{M}$ ) or (B) ICRF 187 ( $200 \mu\text{M}$ ) for 1 hour prior to treatment with PBOX-6 for a further 45 mins. Whole cell extracts were prepared and protein ( $50 \mu\text{g}$ ) was resolved by SDS-PAGE. Blots were probed with anti-JNK phospho antibody, stripped and re-probed with anti-JNK total antibody as a loading control. Results are representative of two separate experiments.





**Fig. 37. PBOX-6 induces apoptosis in MCF-7 cells.**

MCF-7 cells were seeded at a density of  $6 \times 10^5$  cells/ml and were incubated with a range of concentrations of PBOX-6. The control wells in each case contained 0.5% (v/v) ethanol. After 24h the percent apoptosis and necrosis was determined by scraping up the cells, cytospinning and staining them using the RapiDiff kit as described in the Methods section.